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10905 P

Response of Hematopoietic Tissues to Artificially Induced Fever.

MALCOLM M. HARGRAVES, AND CHARLES A. DOAN. (With the assistance of Lucille Kester.)

From the Division of Medical Research, Department of Medicine, Ohio State University, Columbus.

In addition to affording a controlled method for successfully applying therapeutic hyperthermia in certain human diseases, artificially induced fever has proved to be a valuable technic for the study of many fundamental physiologic phenomena. In this laboratory the interest has centered about the effect of radiotherm and hypertherm induced fever on the hemolytopoietic equilibria, first in experimental animals, and, later, in clinical patients.

In a series of rabbits the peripheral blood cell changes following varying degrees of fever for various periods of time have been correlated with the alterations observed in the blood-forming organs. It was found that a leucopenia persisted throughout the duration of the fever induction regardless of the method employed, and only during fever defervescence was leucocytosis of varying degree and duration observed. Furthermore, the leucopenic phase was participated in by all of the white cell elements, the lymphocytes reflecting earlier, and perhaps more profoundly, and certainly more persistently the inhibitory and destructive effects of the fever temperatures. Qualitatively the circulating lymphocytes became increasingly more pyknotic, less basophilic, older appearing cells as the duration of fever was prolonged until, in a rabbit, fevered continuously for 24 hr at temperatures ranging between 108°F and 109°F, they had all but disappeared. Postmortem studies were made of all lymph nodes and of the spleen, and it was found that practically every lymph follicle had disappeared, having been replaced by an infiltration of granulocytes and a great increase in highly phagocytic clasmatocytes with an excess of clear lymph. The sequence of events as reconstructed from a controlled series of animals, in which fever was maintained for varying periods of from 50 minutes to 24 hours, was as follows: lymphocytic nuclear karyorrhexis, cytoplasmic vacuolization, focal cellular necrosis, hemorrhage occasionally, granulocytic infiltration, clasmatocytic proliferation, and inhibition of lymphocytic regeneration with progressive hypoplasia so long as the temperature remained elevated. This is, of course, in conformity with the long recognized hypersensitivity of the lymphatic tissues to many diverse physical and chemical agents, including X-ray, radium, bacterial toxins, etc., and carries further, with added implications, the earlier suggestive experimental observations of Murphy and his associates[1] with dry heat (5 minutes at 55° to 65°C) in mice.

Conversely, however, the circulating granulocytes during this leucopenic period showed a steadily progressive nuclear "left shift", suggesting a constantly maintained delivery of new cells in an effort by the bone marrow to compensate for the increased exodus of polymorphonuclear neutrophils into the tissues under the new conditions. When the rabbit marrows were studied, the chronology of events appeared to be as follows: occasional temporary myelocytic nuclear karyorrhexis was observed during the first hour of fever with more marked megakaryocytic damage, followed promptly thereafter by increased mitotic nuclear figures and myelocytic hyperplasia without progressive necrosis, and therefore with no increase in clasmatocytic activity. Immediately upon reducing the temperature, a granulocytic leucocytosis developed which persisted for some hours. By contrast, only after a considerable latent period of hours did young lymphocytes with deeply basophilic cytoplasm return to the circulation to reflect the gradual regeneration which was having to take place in the lymphopoietic tissues.

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[1] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, **29**, 1.

In those rabbits permitted to survive, the lymph nodes showed the maximum regenerative lymphocytic hyperplasia at the end of 56 to 72 hours post-fever. During 12 repeated feverings at therapeutic levels in the same animal at 3-5 day intervals, the circulating lymphocytes were invariably depressed temporarily while the granulocytes were stimulated, each, however, always returning eventually to the pre-fever equilibrium; and at autopsy no evidences of permanent damage could be demonstrated in any of the hematopoietic organs.

Therapeutic fever of 104°F to 106°F, induced in selected human patients with tertiary syphilis by means of the Kettering Hypertherm, produced a characteristic hemogram when followed by a series of repeated short interval (20 to 30 minutes) blood studies. During the period of fever after a longer or shorter leucopenic phase, a polymorphonuclear leucocytosis developed by tide-like increments of new granulocytes, their direct marrow origin being suggested by a progressive nuclear "left shift", and proved by serial sternal marrow studies. The highest post-fever counts ranged from 10,000 to 60,000 leucocytes at different times and in different individuals. Following the peak, the number of neutrophilic granulocytes decreased more rapidly than the total white count, due first to an increment of regenerating young monocytes and later to returning new lymphocytes. The leucocytic response was independent of the duration of the fever, and occurred following brief, as well as prolonged, temperature elevations. A much less striking granulocytic, nuclear "left shift" and myelocytic marrow hyperplasia accompanied the hypertherm induced leucocytoses than were observed after intravenous typhoid vaccine injections or malaria inoculata given at different times in the same individuals; and the leucopenia was more profound and the succeeding leucocytosis less marked with *B. typhus* and *Plasmodia malariae*.

Due probably to several factors, *e. g.*, anoxia, hyperpyrexic cell damage, and increased metabolic requirements in inadequately prepared and fortified (glycogen, vitamin B_1) tissues, there arises during fever an increased need for granulocytic and clasmatocytic elements in the areas of resultant tissue injury and damage. To this demand the bone marrow promptly attempts response, and to the degree that the supply of available additional cells remains in excess of the increased emigration to the tissues, a peripheral leucocytosis of varying degree occurs without a striking individual nuclear "left shift". If, however, the delivery of new cells is unable to keep pace with the emigration rate to the damaged tissues, a leucopenia with increasingly marked "left shift" develops, and persists until production once again exceeds tissue demands. The much more sensitive lymphocytes are fragmented and destroyed, and their regeneration inhibited at the same fever temperatures.

Conclusion. Artificially induced fever within therapeutic limits destroys lymphocytes and inhibits lymphopoiesis in the normal experimental animal, while at the same time resulting in an increase and extension of marrow myelopoiesis, tissue clasmatocytosis and peripheral granulocytic leucocytosis. The character and nature of this profound effect of fever upon the cellular equilibria may indicate an accessory rôle in the strengthening or weakening of the body defenses—depending upon the particular circumstance—and should be considered whenever artificial hyperpyrexia is contemplated as a means of treating human disease.

10906 P

Bundle-Branch Block and the Monophasic Electrocardiogram.

LINN J. BOYD AND DAVID SCHERF. (Introduced by I. S. Kleiner.)

From the Department of Pharmacology, The New York Medical College, Flower and Fifth Avenue Hospitals.

The accompanying investigations deal with the study of the alterations which are observed in the electrocardiogram when a bundle-branch block is added to a high take-off of the S-T segment and T-wave. Clinical observations have shown that in a recent coronary thrombosis with a high take-off, widening of the initial deflection occurs very rarely; this finding is not adequately explained by the difference in the blood supply of the specific tissues alone.

The combination of bundle-branch block and coronary thrombosis represents an unusual clinical observation.[1] Averbuck and Rachmilevitz[2] have produced a bundle-branch block experimentally after ligation of the branches of the Ramus circumflexus sin. In these experiments the typical electrocardiogram of bundle-branch block did not appear.

A high take-off can be observed not only after ligation of the coronary arteries but also as the result of the influence of certain poisons (potassium chloride, pitressin, digitoxin). We selected *Viscum album* which can produce electrocardiographic alterations in cats and rabbits "suggestive of infarction"[3] when an anal-esophageal lead is employed.

The present experiments were performed on 14 cats and 16 dogs. The animals were anesthetized with nembutal and in a few cases with morphine-nembutal. The electrocardiograms were recorded with the 3 customary leads from the extremities. In the dog experiments after the appearance of the high take-off in the electrocardiogram the right bundle-branch was cut with a slender knife after the thorax and pericardium had been opened. The success of the operation was confirmed at the end of an experiment by autopsy; division of the bundle-branch actually was accomplished in 12 of 16 dog experiments. European *Viscum album* was employed; a 30-50% water extract was prepared by macerating the triturated dried leaves in cold water for 20 hours. One to 2 cc of the extract was injected into the jugular vein every 3-5 minutes. The amount necessary varied according to the size of the animal and the strength of the extract. Naturally these were not uniform.

A high take-off appeared with great regularity. It was very distinct in Leads II and III. Usually it was entirely absent or very indistinct in Lead I. Occasionally in Leads II and III a typical monophasic electrocardiogram appeared. There was no Q-wave and likewise no widening of the initial deflection appeared. The R-wave was lower. Usually sinus rhythm persisted without disturbance but at times an atrioventricular rhythm occurred transiently. Near the end of an experiment disturbances of contraction became distinctly demonstrable, but at autopsy no alterations of tonus of the musculature could be demonstrated. Neither the electrocardiogram nor the disturbance of contractility could be influenced by intravenous injection of aminophyllin or by 50% glucose or by methylene blue. In 4 experiments paroxysmal ventricular tachycardia appeared after aminophyllin.

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[1] White, P. D., *Am. Heart J.*, 1934,**10**, 260.

[2] Averbuck, S. H., and Rachmilevitz, M., *Z. ges. exp. Med.*, 1931, **75**, 562.

[3] Henze, C., and Ludwig, W., *Arch. f. exp. Path. u. Pharm.*, 1937, **187**, 694.

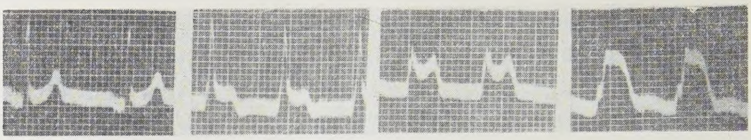

FIG. 1 (Lead II).

Fig. 1 shows the gradual development of a high take-off with transition to a monophasic electrocardiogram. Usually a sinus rhythm was present although at times it was not readily recognizable owing to the remarkably low P-waves; atrioventricular rhythm also appeared transiently. Stimulation of the vagus always produced a distinct depression.

If the right bundle-branch was severed after the appearance of the high take-off, no additional alterations appeared in the initial or terminal deflection or very minor changes became demonstrable as would naturally be anticipated from the progressive effect of *Viscum album*. There was no widening of the initial deflection and the terminal deflection did not become oppositely directed.

In Fig. 2 the first series shows the beginning of the appearance of a high take-off in Leads II and III. In the second series the terminal deflection joins the initial deflection to form a monophasic electrocardiogram. The initial deflection in Lead I has remained small and is directed downward. After the right bundle-branch was severed

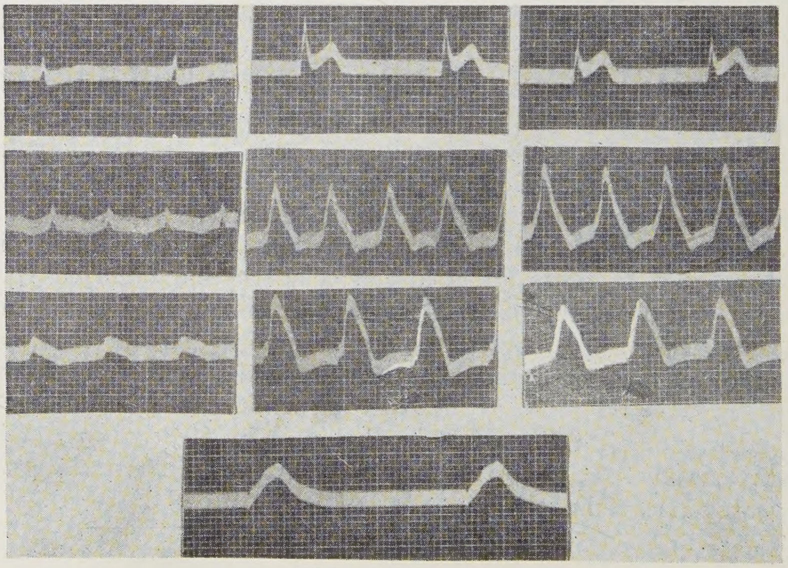

FIG. 2.

(third series) the characteristic changes of a bundle-branch block fail to appear. The lowest tracing shows a progressive widening of the ventricular complex with slowing of the spread of excitation (slow ascent of the R-wave and increasing bradycardia) in the final stage of the intoxication.

If the right bundle-branch was severed at a time when the high take-off was still not distinctly developed, a widening of the initial deflection appeared. With progressive action of *Viscum album* this widening became indistinct.

Fig. 3 shows a right bundle branch block. Examination after the conclusion of the experiment showed that the right bundle branch had been completely severed by 2 parallel cuts. After the bundle had been cut, the intoxication with *Viscum album* was induced. A total of 8 cc of the solution was injected intravenously within a period of 8 minutes. Fig. 3 shows the progressive action of the drug. The S-T segment is displaced increasingly upward and larger sections of

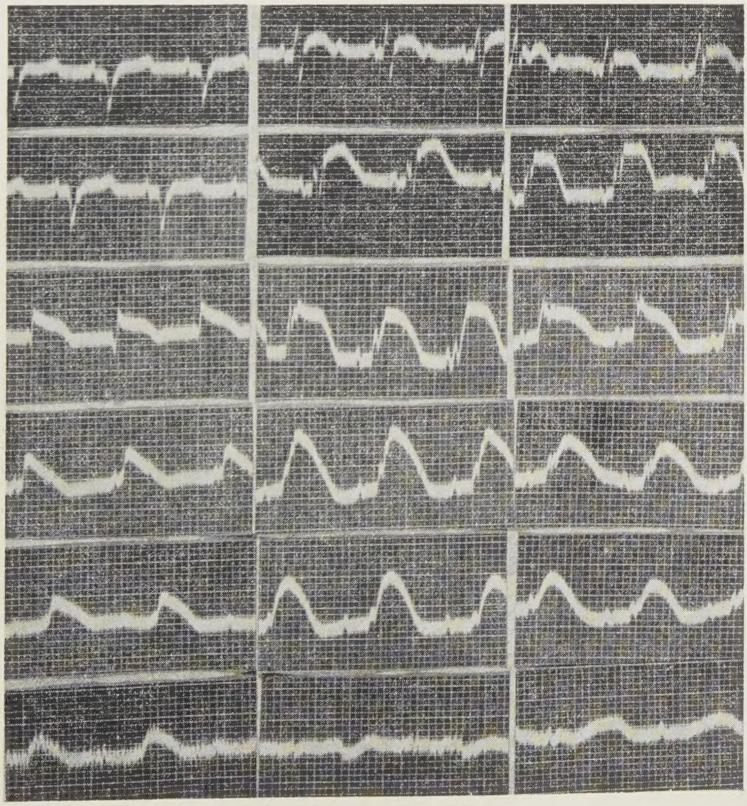

FIG. 3.

the initial complex become included in the S-T segment. In the second series the deep S-waves, produced by the block of the right bundle, are still visible although they are upwardly displaced in the direction of the S-T segment; in the third series they are no longer visible and a monophasic electrocardiogram appears. In the fifth and sixth series conduction from the auricle to the ventricle becomes longer and the slower ascent of the initial deflection which becomes increasingly obvious indicate a slower activation on the ventricle. The initial and terminal deflections can no longer be separated from each other.

Stimulation of the right or left accelerans produces an acceleration of rate but the changes in the terminal deflection, typical of sympathetic stimulation, do not occur.

The high take-off after the administration of *Viscum album* is the result of a profound damage of the muscle tissue itself. No proof was obtained which suggested that a disturbance of blood supply to the heart muscle was involved. We must assume that the activation of the heart is already altered so profoundly by *Viscum album* that the appearance of a bundle-branch block is unable to produce any further changes. Through the appearance of a high take-off and through the incorporation of the terminal deflection into the initial complex, it becomes impossible to demonstrate other alterations of the initial and terminal deflections.

Summary. In cats and dogs *Viscum album* produces a characteristic high take-off. If this is distinctly developed, one can no longer recognize a disturbance of intraventricular conduction (bundle-branch block) and stimulation of the accelerans does not alter the shape of the terminal deflection.

10907

Interdependence of Vitamin B_1 and Manganese. II. Manganese, Copper, Iron Metabolism in B_1 Deficient Rats.

MARTA SANDBERG, DAVID PERLA AND OLIVE M. HOLLY.
From the Laboratory Division, Montefiore Hospital, New York City.

Perla, and Perla and Sandberg[1, 2] in their work on the reproductive behavior of rats have demonstrated a metabolic interdependence of manganese and vitamin B_1. The present study was planned to pro-

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[1] Perla, D., *Science,* 1939, **89**, 132.

[2] Perla, D., and Sandberg, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 522.

vide information on the metabolism of manganese, copper and iron during the development of and recovery from vitamin B_1 deficiency. Since the changes in copper and iron metabolism were not sufficiently constant to suggest a definite rôle of these metals in relation to vitamin B_1, the metabolism of manganese only will be discussed.

Twenty-two male and female albino rats of about 3 months of age were used in the experiment. They were kept in metabolism cages as described previously.[3] Since only negligible amounts of Mn, Cu and Fe are excreted in the urine, feces only were collected twice a week. Feces and food were analyzed for Mn,[4] Cu,[5] and Fe.[6] Blood pyruvic acid[7] was determined weekly in control groups of rats that were kept only for this purpose.

The rats received the Sherman-Spohn vitamin B_1-deficient diet as modified by Chase. This diet contained 6 gamma of Mn per g of food. Additional Mn was given in the form of recrystallized $MnCl_2 . 4H_2O$ of highest quality dissolved in copper-free water. The controls received the same diet, but were supplied with crystalline vitamin B_1.* The rats were kept on the vitamin B_1-deficient diet for a period of 5 weeks. One mg of Mn per rat daily was then added for 3 weeks. When symptoms of vitamin B_1-deficiency were strongly developed, synthetic vitamin B_1 was injected intraperitoneally twice a week, supplying 400 gamma of vitamin B_1 daily, while the addition of Mn was continued. After 4 weeks Mn addition was stopped, but vitamin B_1 was supplied up to the end of the experiment.

The observations of the earlier workers[8, 9, 10] on the effect of Mn on growth may be interpreted to indicate that small amounts of Mn show a favorable effect, possibly because they complement the vitamin B_1 intake, while large amounts may be unfavorable because there is not sufficient vitamin B_1 available to maintain the metabolic balance. This interpretation is supported by our finding that the depletion period of rats on a vitamin B_1-deficient diet was consistently shortened from a week to 10 days if the rats received additions of 1 mg of Mn daily. It has been shown that blood pyruvic acid rises

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[3] Sandberg, M., and Perla, D., *J. Exp. Med.*, 1934, **60**, 395.

[4] Richards, M. B., *Analyst*, 1930, **55**, 554.

[5] McFarlane, W. D., *Biochem. J.*, 1932, **26**, 1022.

[6] Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1926, **67**, 43.

[7] Wilkins, R. W., Taylor, F. H. L., and Weiss, S., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 584.

* We are indebted to the Medical Research Department of the Winthrop Chemical Company for a generous supply of crystalline vitamin B_1.

[8] Richet, C., Gardner, and Goodbody, *Compt. rend. Acad. d. sc.*, 1925, **181**, 1105.

[9] McCarrison, R., *Indian J. Med. Research*, 1927, **14**, 641.

[10] Hamamoto, E., *Orient. J. Diseases of Infants*, 1935, **18**, 21.

TABLE I.

Manganese Metabolism (Daily Average per Rat) During Development of and Recovery from Vitamin B_1 Deficiency

Date, 1938	Experimental Rats				Control Rats			
	Fecal Excret., mg	Intake, mg	Retention, mg	Retention in % of intake	Fecal Excret., mg	Intake, mg	Retention, mg	Retention in % of intake
Sept. 6-12	.068	.084	.016		.056	.069	.013	
,, 13-19	.067	.084	.017		.055	.068	.013	
,, 20-26	.062	.067	.004		.050	.061	.011	
,, 27-Oct. 3	.038	.041	.003		.052	.066	.014	
Oct. 4-10	.027	.045	.017		.035	.043	.008	
Depletion Period Sept. 6-Oct. 10	.053	.064	.011	18	.050	.061	.011	19
Oct. 11-17	.235	1.025	.790		.837	1.048	.210	
,, 18-24	.319	1.023	.704		.775	1.028	.253	
,, 25-31	.288	1.009	.721		.728	1.024	.296	
+1 mg Mn daily Oct. 11-31	.281	1.019	.738	72	.780	1.033	.253	25
Nov. 1-7	.609	1.076	.467		.788	1.024	.236	
,, 8-14	.783	1.103	.320		.797	1.077	.280	
,, 15-21	.754	1.077	.323		.822	1.095	.274	
,, 22-28	.738	1.069	.332		.803	1.070	.268	
+1 mg Mn + 400 gamma vitamin B_1 Nov. 1-28	.721	1.081	.360	34	.802	1.066	.264	25
Nov. 29-Dec. 5	.412	.073	—.340		.420	.057	—.363	
Dec. 6-12	.370	.080	—.290		.301	.059	—.242	
,, 13-19	.674	.080	—.594		.560	.070	—.490	
,, 20-26	.470	.091	—.379		.436	.073	—.363	
+ 400 gamma vitamin B_1 Nov. 29-Dec. 26	.481	.081	—.400		.429	.065	—.364	

in vitamin B_1-deficiency.[11] In our rats the rise in blood pyruvic acid from the initial value of 3.74 mg of pyruvic acid per 100 cc of blood to a peak of 7.28 mg per 100 cc of blood was also observed a week earlier in animals who received additions of Mn to a vitamin B_1-deficient diet.

On a vitamin B_1-deficient synthetic diet the experimental rats stored 18% of the Mn intake, even though this was very low (60 gamma). The controls, which received the same diet but were supplied with crystalline vitamin B_1, stored 19% of the Mn intake. On a Mn intake of 100 gamma daily, contained in a normal diet fed before the start of the experiment, the balance was zero. McHargue[12] suggests that the biological potency of Mn contained in natural foodstuffs differs from that in a synthetic diet. The variation in the ability of our rats to store the Mn of different types of diet may be explained on this basis.

When 1 mg of Mn was added to the diet the vitamin B_1-deficient group stored 72% of the Mn intake while the controls retained only 25%. During the next period, when 400 γ of vitamin B_1 were administered, Mn retention dropped to only 34% of the intake, in spite of the continued high Mn content of the diet. When Mn addition was stopped, the balance became negative, since the excess of Mn which had been stored during the preceding periods was still excreted 4 weeks afterwards, when the experiment was terminated.

10908

Interdependence of Vitamin B_1 and Manganese. III. Manganese, Copper and Iron Metabolism in Normal Rats.

DAVID PERLA, MARTA SANDBERG AND OLIVE M. HOLLY.
From the Laboratory Division, Montefiore Hospital, New York City.

The studies presented in this communication form part of an extended investigation of the metabolic interdependence of manganese and vitamin B_1.[1, 2, 3]

Sixteen male and female rats (Wistar strain) were used. Care of the animals, precautions to avoid unknown Cu intake and methods used in the chemical determinations of Mn, Cu and Fe were the same as those referred to in a previous publication.[3] Feces and food were analyzed for Mn, Cu, and Fe; urine for Mn only.[4] The animals received our stock diet, supplemented with 10% of whole milk powder and 3% of dry brewers' yeast. The Mn content of this diet was 7 γ per g. The rats were studied for a control period of 5 weeks. Then followed 4 periods of one month each, when they received (1) small additions of Mn, (2) large additions of Mn, (3) large additions of Mn plus vitamin B_1, and (4) vitamin B_1. Details about Mn and vitamin B_1 administration are given in the preceding paper.[3] *

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[11] Thompson, R. H. S., and Johnson, R. E., *Biochem. J.*, 1935, **29**, 694.

[12] McHargue, J. S., *Am. J. Physiol.*, 1926, **77**, 245.

[1] Perla, D., *Science*, 1939, **89**, 132.

[2] Perla, D., and Sandberg, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 522.

[3] Sandberg, M., Perla, D., and Holly, O. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 368.

Since changes in Fe metabolism were not significant, protocols for Mn and Cu metabolism only are presented.

During the control period when the rats were given a normal diet, supplying about 100 γ of Mn daily, they did not store any Mn. When, however, the Mn content of the food was raised about 50%, 25% of the intake was retained. The addition of 1 mg of Mn per g of food, though raising the intake to over 13 mg of Mn, did not change the percentage of retention. If, however, 400 γ of vitamin B_1 daily were given, the large amount of Mn offered in the diet was utilized to a greater extent. We have found[3] that in vitamin B_1 deficient rats administration of vitamin B_1 caused a drop in the retention of Mn to about half the amount retained during development of the avitamino-

TABLE I.
Manganese Metabolism (Daily Average per Rat).

Date	Urine, mg	Feces, mg	Total Excretion, mg	Intake, mg	Retention, mg	Retention in % of intake
Control period Mar. 8-Apr. 11	0.002	0.099	0.101	0.096	—0.005	0
+ 5 γ Mn/g of food Apr. 12-May 9	0.002	0.122	0.124	0.165	0.041	25
+ 1 mg Mn/g of food May 10-June 6	0.005	10.061	10.066	13.487	3.421	25
+ 400 γ of vitamin B_1 + 1 mg Mn/g of food June 7-13	0.005	8.049	8.054	13.640	5.586	41
+ 400 γ of vitamin B_1 + 1 mg Mn/g of food June 14-July 4	0.005	6.837	6.842	14.819	7.977	54
+ 400 γ of vitamin B_1 July 5-11	0.003	1.791	1.794	0.088	—1.706	—
+ 400 γ of vitamin B_1 July 12-25	0.002	0.113	0.115	0.102	—0.013	0

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[4] Richards, M. B., *Analyst*, 1930, **55**, 554.

* We are indebted to the Medical Research Department of the Winthrop Chemical Company for a generous supply of crystalline vitamin B_1.

sis. Normal rats on a normal diet are enabled to store a greater part of an unusually high Mn intake if large amounts of vitamin B_1 are offered at the same time. Apparently this does not represent a mere retention of inert material as in vitamin B_1-deficient animals, where it is excreted again when administration of vitamin B_1 permits a normal functioning of the organism. In normal animals only a small part of the accumulated Mn was excreted during the first week when Mn addition was stopped while administration of vitamin B_1 was continued. In the following weeks when Mn intake amounted again to about 100 γ, continued vitamin B_1 administration did not influence the retention of Mn which was practically zero, just as in the control period.

Since Cu is recognized as an oxidative catalyst it was considered possible that a change in Mn metabolism might show an influence on Cu metabolism.

During the control period when the Mn balance was zero, 30% of the ingested Cu was retained. When Mn retention had been raised to 25% of the intake by addition of Mn to the ration, Cu retention dropped to as low as 15% of the intake. This suggests the possibility that if large amounts of Mn are available, less Cu may be required for oxidative processes in which one or the other of the trace metals plays a part. The administration of 400γ of vitamin B_1, while raising Mn storage to 41% of the intake, did not influence the low level of Cu retention during the first week. After that Cu retention rose to 21% of the intake. Since Mn retention amounted to 54% of the intake during this period it may be that large amounts of Mn and vitamin B_1, if offered simultaneously, may permit a better utilization of Cu as well as of Mn. When Mn addition was stopped while vitamin B_1 administration was continued, Mn retention became negative and Cu retention dropped again to 15% of the intake for one

TABLE II
Copper Metabolism (Daily Average per Rat)

Date	Feces, γ	Intake, γ	Retention, γ	Retention in % of intake
Control period Mar. 8-Apr. 11	52	74	22	30
+ 5 γ Mn/g of food Apr. 12-May 9	59	73	14	18
+ 1 mg Mn/g of food May 10-June 6	66	77	11	15
+ 400 γ of vitamin B_1 + 1 mg Mn/g of food June 7-13	64	76	12	16
+ 400 γ of vitamin B_1 + 1 mg Mn/g of food June 14-July 4	65	83	18	21
+ 400 γ of vitamin B_1 July 5-11	63	74	11	15
+ 400 γ of vitamin B_1 July 12-25	62	86	24	28

week. During the following weeks both Mn and Cu retention returned to normal proportions. Continued administration of vitamin B_1 failed to influence Cu metabolism as long as the Mn intake remained at the normal level of 100 γ. The Cu intake of about 70 to 80 γ daily remained unchanged throughout the experiment.

10909

Relation Between Latent Period and Growth Rate in Chemically Induced Tumors.

AUSTIN M. BRUES, ALBERT E. WEINER AND HOWARD B. ANDERVONT. (Introduced by Joseph C. Aub.)

From the Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, and the Office of Cancer Investigation, U. S. Public Health Service.

It has been customary to rate the potency of carcinogenic action according to the shortness of the latent period or the percentage of animals developing tumors (Boyland and Warren,[1] Cook, *et al.*[2]). It is of interest to know whether malignancy of the tumors produced is correlated with the potency of carcinogenic action as measured by the latent period. Of the various criteria of malignancy, it has seemed to us most appropriate to investigate growth rate as measured directly *in vivo,* or indirectly by means of mitosis counts.

The mice in these experiments, representing various inbred strains, were injected in the subcutaneous tissues of the right axilla with carcinogenic agents dissolved in lard or in cholesterol pellets. The mice were examined biweekly and the latent period was reckoned from the time of injection to the time when tumors were first noted by palpation. Tumors were then measured biweekly in 2 dimensions by a single observer, who recorded the diameters to the nearest millimeter, using calipers with approximately constant pressure. Growth rate was estimated in terms of the increase in the mean of these diameters per week of observation. Only those tumors were included in the series in which at least 4 observations covering a period of 10 days or more were made. Most mice were killed after tumors had reached considerable size and paraffin sections were made. Mitoses were enumerated in groups of 1000 or more counted tumor cells. The findings are recorded in Table I.

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[1] Boyland, E., and Warren, F. L., *J. Path. Bact.*, 1937, **45**, 171.

[2] Cook, J. W., Hieger, I., Kennaway, E. L., and Mayneord, W. L., *Proc. Roy. Soc. London*, 1932, **3**, 455.

TABLE I.
Mean Latent Period, Growth Rate, and Mitosis Rate.

Strain of mice	No. of mice		Agent	Vehicle	Mean latent period, wk	Growth rate, mm per wk	Mitoses. % of tumor cells
C3H	37	2	mg MCA	lard	8½	4.68±.25	.96±.35
A	15		" "	"	10½	4.42±.58	
RPE*	22		" "	"	15	3.00±.11	.70±.36
I	20		" "	"	16	2.82±.50	.70±.26
C3H	37		" "	"	8½	4.68±.25	
"	15	1	" "	"	9½	3.78±.27	
"	36	0.5	" DBA	"	20	3.02±.30	
C57—black	22	0.45	" MCA	Cholesterol	12	3.01±.31	
"	6	0.45	" DBA	"	26	1.06±.71	

* Roberts Pink-Eye.

It will be seen that with the same carcinogenic technic, those strains of mice which produce tumors slowly, develop slowly-growing tumors. When the dosage or agent applied to a single strain is varied, we see the same correlation between speed of response and growth rate. In fact, under the various conditions of these experiments, there is in all cases a high direct correlation between growth rate and shortness of the latent period. No significant differences in growth rate were seen between the early and late appearing tumors of a single series, due perhaps to the small numbers of animals in the series.

In the 3 groups in which mitosis counts were done, they were seen to show a good degree of correlation with growth rate. Here, again, it was not possible to demonstrate such correlations in individual instances, but only by groups.

The question naturally arises as to what extent the latent period is determined by the growth of a tumor which has not yet reached visible size. A rough idea of the magnitude of the period of tumor development can be obtained by the help of a few simplifying assumptions. The mean duration of mitosis in a tumor cell has been estimated as at least one hour (Lewis and Lewis,[3] Lewis[4]), or slightly more than in normal tissues (Brues and Marble[5]). On this basis the proportion of cells in mitosis represents approximately the hourly percentage increase in tumor size. If we make the reasonable assumption that growth rate remains constant without retarding influences such as are seen in embryos and regenerating tissues (Brues

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[3] Lewis, W. H., and Lewis, M. R., *Am. J. Cancer*, 1932, **16**, 1153.

[4] Lewis, W. H., *Am. J. Cancer*, 1939, **35**, 408.

[5] Brues, A. M., and Marble, B. B., *J. Exp. Med.*, 1937, **65**, 15.

TABLE II.

% cells in mitosis	Days required for tumor to double in size (intermitotic period)	Days required for tumor to increase from 10^{-9} to 0.1 cm³	Days required for tumor to increase from 10^{-6} to 0.1 cm³	Actual latent period (days)
0.70	4.1	109	70	105
0.96	3.0	80	50	60

and Marble[5]) the growth should follow an exponential curve ($v = v_o \times e^{kt}$) when v and v_o are final and initial tumor volumes after a lapse of time t, and k is a constant determined by instantaneous growth rate and reflected in the mitotic index. On this basis Table II has been constructed to show the "latent periods" in the development of a tumor of 0.1 cm³ from a single cell and from 1,000 cells (estimated as 10^{-9} and 10^{-6} cm³, respectively), with growth rates represented by 0.96 and 0.70% per hour. The latent periods actually seen in the development of tumors with similar mitotic rates (C3H and RPE strain) are shown alongside those so calculated.

Thus, if we suppose that neoplasia begins with a relatively small number of malignant cells and follows a constant growth rate, a great part of the latent period is involved in the growth of a microscopic tumor to palpable size. This supposition is confirmed by our observations that the great majority of these tumors are made up of homogeneous cells, although cell type varies widely from one tumor to the next.

Similarly, when the development of spontaneous mammary tumors is inhibited, palpable tumors continue to appear for 4 months (Nathanson and Andervont[6]). The median growth rate of a series of mammary tumors in mice has been shown to be less than 3 mm per week (Haddow[7]). It seems significant, therefore, that those induced tumors in our series which show a parallel growth rate (I strain mice) have a latent period of 16 weeks. In any case, it is probable that the latent period is greatly influenced by the growth rate.

When a constant dose of carcinogen is used on various strains of mice, the parallel variations in growth rate and latent period may well be due to quantitative differences in response of the individual cells, reflected in different tumor growth rates. It has been observed that these several strains of mice respond also with a tendency to develop different types of tumor (Brues and Andervont, unpublished data). When various types and dosages of carcinogen are used, however, variations in latent period might be due to differences in the period before initial carcinogenesis, or in the number of malignant cells originally produced.* But the correlation between latent period and growth rate suggests that here, too, growth rate is an important factor. The short latent period in the induction of tumors by virus would necessarily be explained by the fact that large numbers of malignant cells are produced simultaneously. These observations are consistent with those of Mottram[9] that latent periods and growth rates of tar warts and epitheliomata extrapolate in the same way.

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[6] Nathanson, I. T., and Andervont, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 421.

[7] Haddow, A., *J. Path. Bact.*, 1938, **47**, 553.

Summary. There is a high degree of correlation between the malignancy of chemically induced tumors, as measured by growth rate and mitotic index, and shortness of the latent period before appearance of palpable tumors. This relation holds true in the various responses of different strains of mice to the same agent, and in responses to different agents and modes of administration.

It is suggested that the growth rate of a tumor in the microscopic stage is important and may be the main factor determining the latent period in carcinogenesis.

10910 P

Observations on the Specific Cause and the Nature of "Quail Disease" or Ulcerative Enteritis in Quail.*

CHARLES C. BASS.

From the Laboratory of Clinical Medicine, School of Medicine, Tulane University of Louisiana, New Orleans.

Ulcerative enteritis of quail (so-called quail disease) constitutes the most important disease problem in propagation of quail in captivity. Many game breeders have had their entire stock decimated or practically wiped out by an epizootic of this disease. Once introduced on a game bird farm, the infection is extremely difficult to eradicate. Heavy losses from this disease have occurred in wild trapped quail

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* Similarly, it is well known[8] that the latent period after transplantation of tumors depends on the number of tumor cells inoculated.

[8] Schrek, R., *Am. J. Cancer*, 1936, **28**, 364.

[9] Mottram, J. C., *J. Path. and Bact.*, 1935, **40**, 407.

* This work has been aided by a grant from Mr. A. B. Freeman and Mr. Robert Newman, of New Orleans.

held in concentration stations for restocking purposes. This indicates that the disease may profoundly affect the population density of wild quail in their natural habitat.

The purpose of this communication is (a) to record the previously unrecognized† specific cause of quail disease, (b) to describe the technical method by which others can readily confirm this, (c) to direct attention to the chronic carrier as a factor in maintaining and spreading the infection and (d) to record observations indicating transmission of the infection to young quail chicks through eggs laid by carriers.

The disease is characterized by numerous lentil-shaped intestinal ulcers located mostly in the lower third of the ilium and less numerous in the caeca. They vary in size from pin-point to half the circumference of the intestine or even larger. The small pin-point lesions are buried deep in the mucous membrane, between, and involving, the villi. Larger lesions present an open, crater-like ulcer, with very much thickened base.

In microscopic sections through the very small lesion one finds it to consist of a mass of necrotic tissue, containing large numbers of Gram-negative bacilli which can be seen to be invading the surrounding living tissue. In sections through larger lesions with open ulcers one finds this same Gram-negative bacillus and also many other secondary organisms, especially near the surface. If a small unbroken lesion (necrotic plug) is teased out, crushed on a slide and stained, one always finds large numbers of the characteristic organism. Occasionally spores can be found on slide preparations made direct from the lesions.

I have isolated this organism in pure culture 7 times—5 from intestinal lesions, one from a similar very small metastatic lesion in the liver and one from the yolk-sac of a baby quail. It is strictly anaerobic and grows slowly. The best growth I have obtained has been in glucose agar (0.25% agar) to which was added a small amount of an aqueous extract of macerated quail intestine (lower third of ilium). Oxygen must be driven off by boiling the medium before it is inoculated. In properly inoculated tubes of such medium the organism grows, in very small colonies, to within about 1 cm of the surface.

The best technic I have used for demonstrating the specific organism is as follows: (a) Kill a sick bird, (b) clip out a piece of intestine containing a very small lesion that has not ulcerated into the lumen, (c) wash this tissue by shaking it vigorously in salt solution, (d) spread it out on a slide or glass plate, (e) with 2 teasing needles dig out the small greyish plug, (f) place it on a slide and crush and spread with the end of another slide, (g) fix with heat, and (h) stain by the Gram technic.

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† L. C. Morley and P. W. Wetmore, in a brief report published in the *Proceedings, North American Wildlife Conference*, Feb. 3-7, 1936, described a Gram-positive corynebacterium which they considered to be the cause of quail disease. We have often found the organism which they described, in open ulcerated lesions, but it is a secondary invader and is not the specific cause of the disease.

In carefully controlled experiments I have transmitted the disease to clean birds by feeding (1) droppings from sick birds, (2) macerated diseased intestine, (3) macerated remains of the yolk-sac (in which the bacilli had been found) of a 19-day-old quail, and (4) pure cultures of the organism; also, by placing clean birds in contaminated cages with, or just after the removal of diseased birds. Death in such infected birds usually occurs within a few days, especially if they are young birds (half grown or younger).

Of a large number of adult birds infected in my laboratory about January 1, 1939, about 75% died during the first 2 months, about 15% during the third month and about 4% during the fourth month. A few live still longer. During the entire period such birds are carriers and potential sources of infection to other birds.

Several of the known carriers laid eggs during the time and were found to have the characteristic lesions and organism when they died. In 3 instances birds laid within 48 hours before death, one of them within 2 hours. There is good opportunity for the egg to become infected during its development in the bird or to become contaminated from the feces when it is laid.

I have found the disease and confirmed the diagnosis by the presence of ulcers and the specific organism in one 10-day-old bird and in one 9 days old. In a 5-day-old bird I found acute inflammation in the section of the ilium where lesions of quail disease are found, and also found an abundance of Gram-negative bacilli that resembled the specific organism of this disease. This intestine was macerated and fed to a clean 10-weeks-old bird. It died on the fifth day and autopsy showed typical acute quail disease and the specific organism. This 5-day-old bird had been kept in a new clean pen since it was hatched and must have acquired its infection from the egg.

In 2 instances, one a 14-day-old quail and the other a 19-day-old bird, dead of quail disease, I have found the characteristic Gram-negative organism in a small encysted tumor of the remains of the yolk-sac. The bacilli were arranged in small masses or clumps within the tumor mass and gave the appearance of very small colonies that had grown there. The organism was isolated in pure culture from one of the yolk-sacs (the 14-day-old bird) and the disease was produced experimentally in a clean bird by feeding a part of the material direct from the other. Although I have not been able to find the specific organism in an egg before hatching, the observations recorded above indicate that very young birds acquire quail disease through eggs infected with the specific organism and layed by carriers of the infection.

10911

Procaine Base Dissolved by Means of CO_2 and Its Mode of Action.*

R. BEUTNER.

From the Department of Physiology and Pharmacology, University of Louisville School of Medicine, and the Department of Pharmacology, Hahnemann Medical College.

It is well known that the local anesthetic power of procaine hydrochloride or related local anesthetics is augmented by the addition of sodium bicarbonate or other alkaline salts. This effect is readily explained through the stronger action of the uncombined procaine base which is liberated from the salt by sodium bicarbonate, etc. However, only an unknown part of the procaine base is thus liberated since chemical equilibrium exists between procaine HCl and sodium bicarbonate and the resultant products.

The use of procaine base as such in a water soluble form became possible through the observation that procaine loosely combines with CO_2, thus being rendered water soluble, probably as a carbonate. On account of the rapid diffusion of CO_2 in the tissues, this solution can be expected to act as if the procaine base was dissolved as such.

Such a solution of procaine can be made as follows: 2 to 5 g of procaine base are suspended in 100 cc of water which is warmed to, or very slightly above, the melting point of the base (59°C). A steady stream of CO_2 is conducted through the mixture which is shaken vigorously. Further heating is avoided so long as the procaine is still a liquid, since above 60°C the solubility of CO_2 in water is too slight. Should procaine crystals appear, the suspension is reheated to the melting point. CO_2 is rapidly absorbed and the procaine goes into solution.

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* This investigation has been partly made with the assistance of a grant from the Committee on Therapeutic Research, Council of Pharmacy and Chemistry, American Medical Association.

With a procaine solution prepared in such a manner, the following experiments were performed by Dr. H. Wastl† at the suggestion of the writer:

(1) The incidence of convulsions in guinea pigs was tested. As previously shown by Beutner and Miley,[1] guinea pigs usually survive procaine and other convulsants, the incidence of convulsions being indicative of the convulsive power of any given local anesthetic. From Dr. Wastl's observations the following data were figured through graphic interpolation:

100 mg per kg procaine base	convulsions in all injected animals (136 of them) (direct observation)
100 mg per kg procaine as hydrochloride	convulsions in about 87% of the injected animals (calculated by graphic interpolation)
50 mg per kg procaine base	22.2% convulsions
50 mg per kg procaine as hydrochloride	22% convulsions

It appears, therefore, that the central convulsive action of procaine is not very different whether it is dissolved as a hydrochloride or, in looser combination, by means of CO_2. Evidently while passing through the blood stream, procaine is liberated nearly to the same extent no matter whether it is combined with HCl or with CO_2.

(2) In another series of experiments the local anesthetic effect on the cornea of the rabbit was tested. It was found to be much greater for procaine dissolved by CO_2 than for procaine hydrochloride. This is to be expected in view of the scarcity of buffer material present on the cornea. It was found that a solution of procaine hydrochloride containing 0.5% of the base when instilled on the conjunctiva anesthetized in 4.5 minutes (average of 6 observations). When procaine was dissolved in CO_2 at the same concentration, anesthesia occurred much faster, *viz.*, in 1.2 minutes (average of 6 observations). With 0.25% procaine as hydrochloride anesthesia occurred in 5.6 minutes (average of 6 observations); with 0.25% procaine dissolved in CO_2, in 3.8 minutes (average of 6 observations).

As to be expected, these figures show that the topical effect of procaine CO_2 combination is considerably larger than that of the hydrochloride and corresponds to that of the free base.

Summary. A method of dissolving procaine base by means of CO_2

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† The writer wishes to thank Dr. H. Wastl for her careful observations from which the above figures are calculated.

[1] Beutner, R., and Miley, G. P., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 279. Compare also Beutner, R., and Wastl, H., *Scientific Proc. Am. Soc. for Pharm. and Exp. Ther.*, Thirtieth Annual Meeting, Toronto, Ontario, Canada, April 26-29, 1939.

is presented. The solution thus formed is only slightly more convulsant than procaine hydrochloride but considerably more anesthetic on topical application.

10912

Effect of a High Fat Diet on Carbon Dioxide Combining Power of Blood Plasma.

HELEN L. WIKOFF AND JOSEPH F. VINCENT. (Introduced by Clayton S. Smith.)

From the Department of Physiological Chemistry, College of Medicine, The Ohio State University, Columbus, Ohio.

Stewart, Gaddie and Dunlop[1] in a series of experiments on human subjects studied the carbon dioxide combining capacity of plasma with relation to blood fat content during and after exercise. They found that total lipid content varied inversely with the blood carbon dioxide combining power. These investigators believed that the carbon dioxide combining power was responsible for the changes in the blood fat content because changes in carbon dioxide combining capacity preceded the changes in fat content.

Since the subjects were not fed a high fat diet, nothing can be learned concerning the effect of an excessive amount of fat in the plasma on the CO_2 combining power. Van Slyke, Sendroy, Hastings and Neill[2] have shown that carbon dioxide is much more soluble in lipemic plasma than in normal plasma. Accordingly, even if the alkaline reserve of a lipemic blood sample were decreased, this might not be apparent from the carbon dioxide combining capacity as ordinarily determined from whole plasma.

During the past year, a group of investigators at this university conducted an experiment in which they fed students meals consisting chiefly of fried foods and pastries. Since such a diet is obviously high in fat content, we believed that information concerning the influence of fat in the diet on the carbon dioxide combining power of plasma might be obtained by studying the blood of these subjects. Permission was therefore granted us to draw the samples necessary for conducting the present investigation.

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[1] Stewart, C. P., Gaddie, R., and Dunlop, D. M., *Biochem. J.*, 1931, **25**, 733.

[2] Van Slyke, D. D., Sendroy, J., Jr., Hastings, A. B., and Neill, J. B., *J. Biol. Chem.*, 1928, **78**, 784.

Three groups of university students were observed consecutively for periods of 6 weeks each. Each group consisted of 10 men and 10 women. The ages of the subjects varied from 17 to 27 years. The heights varied from 65 to 73 inches for the men, and from 59 to 70 inches for the women. The men weighed from 123 to 180 pounds; the women from 79 to 188 pounds. As these data indicate, short, tall, thin, and fat individuals as well as those of so-called average stature were included in the groups.

The subjects were selected to include as nearly a representative cross-section of the university student body as possible. Consequently their daily activities varied as markedly as did their physical characteristics. The only factor common to the entire group was the type of food eaten during the 6 weeks' duration of the experiment. Each subject could eat as much as desired at the meals provided, but no other food or meals were allowed to be eaten elsewhere during the 6-week interval.

During the first week of the experiment, a normal well-balanced diet was provided for each group. During the second and third weeks of the experiment, an adequate diet having a high fat content was served. The diet during the fourth week was essentially the same as that of the first week, while the menus of the second and third week were duplicated during the fifth and sixth week.

The fresh foods and the vegetables served varied with the season of the year. Meals were served to the first group during October and part of November. Group II ate the special meals during the first 3 weeks in December and after a 3-weeks interval resumed the meals in January for another period of 3 weeks. Group III was fed during February and part of March. The season when these experiments were conducted is the time of the year when most individuals increase their fat intake.

Since our experiment was not really a part of the original investigation designed to duplicate home conditions as far as possible, it was not feasible for us to weigh the amounts of food eaten by the subjects nor to analyze it. However, at all times we had access to the menus showing the foods eaten by the subjects. Expert dietitians had previously checked the menus to make sure that adequate amounts of all essential foodstuffs were always provided in addition to the excessive amount of fat. During the test periods, about 2 pounds of fat per week per person were used in the preparation of the foods but some of this, a portion of the fat used in deep frying was discarded from time to time.

Two or 3 weeks before the feeding experiments began, a preliminary sample of blood was obtained from each member of Groups II and III. The blood drawn from the median vein of the forearm was immediately oxalated, and then centrifuged. The plasma, so obtained, was poured into paraffined tubes and stored in a refrigerator. When the subjects were on the experimental diets, blood samples were drawn on the days preceding each change in diet.

The CO_2 combining capacity of each sample was determined by the usual Van Slyke manometric procedure.[3] Analyses were all made within 8 hours after the drawing of the samples in the case of Groups II and III. Some plasma samples obtained from members of Group I stood for 3 days before the analyses were made.

Table I shows the carbon dioxide combining capacities (expressed as volumes percent) for the members of Groups I, II and III respectively. We were unable to draw blood from one of the women subjects in Group I, consequently only 19 sets of values are listed for Group I. Two of the men in Group II dropped out at the end of the first 3 weeks and were replaced by other men. Incomplete analyses for 12 men subjects are therefore included in the table. Occasionally a subject forgot to report at the proper time for drawing blood samples as is evidenced by blank spaces in the table.

Discussion. Inspection of Table I fails to reveal any common reaction to a high fat diet. Some individuals show a marked deviation from the CO_2 content of plasma drawn under normal conditions of diet while others apparently are not affected at all. Even the deviations from normal values show no constant trend. Independent individual variations are caused by such factors as exercise, state of health, metabolic rate, inherent ability to metabolize fats, and amount of fat in the blood stream.

When the averages of the individual findings for the men and the women of each group are studied, it may be seen that (with the exception of the second control diet of Group II) the carbon dioxide combining capacities of the men are all higher than the corresponding values for the women. This is also true for the general averages for all men and all women.

If these average results are still further investigated, it will be seen that the average values for the carbon dioxide combining capacities of the men in Groups I and II on the first high fat diet are higher than values obtained while the same subjects were on the control diet. However, during the latter half of the experiment, the average values for the same subjects are lower on the high fat diet than when on the control diet. The behavior of the average carbon

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[3] Van Slyke, D. D., *J. Biol. Chem.*, 1917, **30**, 347.

TABLE I.
The CO_2 Combining Capacities Expressed in Volumes Percent.

Group I Subject No.	CO_2 Combining Capacity (Volumes %) Pre-Control				
	Pre-Control	Control Diet	High Fat Diet	Control Diet	High Fat Diet
Men					
1		72.2	63.2	57.2	67.0
2		61.3	62.5	61.0	63.2
3		64.8	64.0	67.0	68.0
4		71.9	68.0	63.3	63.8
5		68.3	63.9	67.0	70.0
6		70.0	69.5	61.0	65.8
7		68.3	66.5	57.8	65.2
8		66.5	65.3		64.5
9		74.7	65.6	70.9	65.2
10		56.1	61.4	63.0	68.8
Avg		67.6	65.0	63.1	66.2
Women					
11		64.0	64.5	65.0	
12		53.7	58.8	51.0	59.3
13		57.1	64.1	64.0	67.0
14		64.0	65.8	55.2	61.8
15		55.9	65.1	58.0	62.0
16		64.0	52.0	66.2	49.0
17		65.6		56.0	60.9
18		60.0	61.1	60.7	51.0
19		60.9	66.5	56.8	61.0
Avg		60.6	61.0	59.4	59.0
Group I avg =		64.4	63.2	61.4	63.0
Group II					
Men					
20				57.8	58.0
21	62.1	68.7	67.0	66.5	66.0
22	64.0	66.2	62.5	64.0	65.0
23	64.5	72.8	77.5	62.5	
24	69.0	70.5	75.0	69.5	67.0
25	64.5	64.2	70.5		
26				60.8	66.5
27	71.0	66.0	70.0	59.0	61.0
28	67.8		56.0		
29		66.5	67.5	59.0	
30		72.5	68.0	57.0	67.7
31		66.2	52.0	69.5	65.5
Avg	66.0	68.1	66.0	62.6	64.7

Group II (cont'd) Subject No.	CO_2 Combining Capacity (Volumes %)				
	Pre-Control	Control Diet	High Fat Diet	Control Diet	High Fat Diet
Women					
32	59.0	54.0	65.5		
33	68.0	61.0	65.0	69.5	62.8
34		64.5	61.0	65.5	62.9
35	63.1	60.8	64.4	59.0	63.5
36	66.0		65.5	69.5	65.6
37	58.0	61.4	66.0	65.0	51.9
38	59.1	65.2	63.2	61.5	58.5
39	63.0	66.7	59.0	64.7	60.5
40		58.4		62.5	61.0
41		64.5	62.0	60.8	59.5
Avg	62.4	61.9	63.5	65.7	60.4
Group II Avg =	64.0	65.1	61.7	64.0	62.5
Group III					
Men					
42	61.7	62.0	63.0	58.5	53.0
43	67.0	65.5		61.0	64.2
44	67.5	57.0	62.8	58.0	61.9
45	71.5	65.7	66.7	62.8	65.0
46	68.0	66.4	67.0	57.2	67.5
47	66.5	61.4	66.4	60.2	68.2
48	66.0	68.0	65.0	69.1	67.0
49	70.5	61.6	64.0	65.4	65.2
50	72.0	65.0	61.0	68.0	65.0
51	67.5	49.5	66.0		61.6
Avg	67.8	62.2	64.7	62.2	63.8
Women					
52	60.5	56.5	59.2	63.5	64.0
53	62.5		65.0	57.8	59.4
54	55.8	61.3	61.9	61.0	65.0
55	64.2	62.7	60.0	58.5	62.2
56	61.7	62.5	56.7	60.2	63.8
57	63.0	61.2	61.9	54.3	62.3
58	52.9	59.5	59.4	54.7	58.5
59	62.9	63.2	62.1	59.2	64.3
60	60.5	64.0	60.5	55.2	61.8
61		63.1	57.0	63.0	53.4
Avg	60.5	61.5	60.4	58.7	61.5
Group III Avg =	64.4	61.9	62.5	60.4	62.5
Average for all men =	66.9	66.0	65.2	62.6	64.9
" " " women =	61.4	61.4	61.8	61.3	60.5
" " " subjects =	64.2	63.8	63.8	61.9	62.7

dioxide capacities for the women subjects, eating with the same groups is exactly the reverse of that of the men. The values increase on the first high fat diet and decrease on the second. The values for the men in Group III behave like those for the women in

Groups I and II, while those for the women in Group III behave like those for the men in Groups I and II. It is apparent that no striking conclusions can be drawn from these data.

If the individual findings for the various groups are again studied and if variations of 2 to 3 volumes per cent are regarded as experimental error, a table (Table II) may be constructed showing the percentage of total subjects in which the carbon dioxide combining capacity increases, decreases, or does not vary while on the high fat diet. In each case, the comparison is made between the value obtained during the high fat diet and that for the control period immediately preceding.

TABLE II.
Percentage of Total Subjects Showing Changes in CO_2 Combining Capacities When Fed High Fat Diets.

	Increase %	Decrease %	No change %
High Fat Diet No. 1	28.2	26.4	45.6
” ” ” ” 2	41.6	22.6	35.8

Table II shows that there are nearly as many persons who experienced no changes in CO_2 combining capacity while on the first high fat diet as there are persons who did exhibit deviations. These variations are about equally divided between increases and decreases. However, when the second high fat diet was fed, a considerable greater percentage of subjects showed a deviation from normal and this variation is definitely towards an increased CO_2 combining capacity. However, it is our opinion that this is an apparent rather than true increase in carbon dioxide combining capacity and is caused by the increased solubility of carbon dioxide in lipemic plasma.[2]

Summary. 1. A high fat diet produced no characteristic reaction as measured by the carbon dioxide combining capacity of blood plasma. 2. The carbon dioxide combining capacities of the men were greater than those of the women when on identical diets.

10913

A Polarographic Study of Insulin.*

OSCAR KANNER AND G. J. REED. (Introduced by E. M. K. Geiling.)
From the Foundation for Dental Research of the Chicago College of Dental Surgery.

Polarographic features of insulin were first studied by Brdicka.[1] Later, Mulli and Werner,[2] and Tropp[3] also made polarographic studies of insulin. Mulli and Werner claimed that they could determine the activity of any sample of insulin from the polarographic curves. Tropp, who made a more substantial study of the problem, maintains that it is possible to compare activities and height of polarographic steps of a given brand of insulin.

The experiments to be reported here show that the height of polarographic steps is solely a function of the SS or SH concentration of the insulin; it may be independent of its activity. This investigation of insulin is based on 4 assumptions: (1) that it is possible to determine polarographically the concentration of SS and SH groups of a protein, (2) that this concentration is approximately proportional to the protein concentration if similar proteins are concerned, (3) that the insulin solutions contained no elements that interfered with the development of the characteristic steps, and (4) that the activity of the tested solutions of insulin corresponded to the data given by the manufacturer.

The first assumption is justified if a constant fraction of the SS groups present in insulin is polarographically evident. The second assumption presupposes that all insulins contain nearly equal proportions of polarographically active SS groups.

In any given case it is possible to verify assumption three because experience has shown that substances tending to suppress the characteristic waves may be practically eliminated by appropriate dilution.

The polarograph is an instrument for automatic registration of current-voltage curves obtained by electrolysis of the investigated substance with a dropping mercury cathode and an unpolarizable mercury anode. Cations, reducible molecules, or catalytic sub-

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* Read before the Annual Meeting of The Federation of American Societies for Experimental Biology, Toronto, Canada, April 27, 1939.

[1] Brdicka, R., *Collection of Czechoslovak Chemical Communications*, 1933, **5**, 112.

[2] Mulli and Werner, *Deutsche Med. Wochenschr.*, 1937, **63**, 1941.

[3] Tropp, C., *Klin. Wochenschr.*, 1938, **17**, 465.

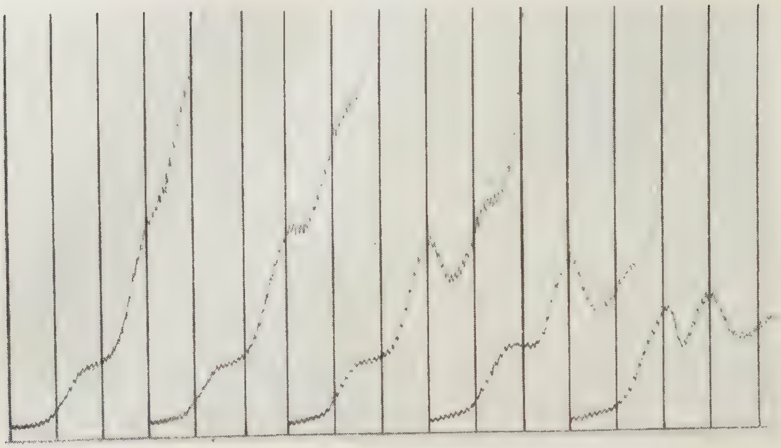

FIG. 1.

Solutions of crystalline insulin in concentrations 20 mg%, 10 mg%, 5 mg%, 2.5 mg%, 1.2 mg%.

The first step of each curve indicates cobalt, added to induce the catalytic protein steps. The second double step indicates protein. Galv. sens. 1/200.

stances are characterized by the voltage at which steps occur on the current-voltage curve. Their concentration is indicated by the height of these steps.† Confirming Brdicka, it was observed that insulin yields curves which are not distinguishable from those of other polarographically active proteins. With available methods, polarographic determination at best gives only an approximation of the protein contents of any given sample of insulin.

The insulin step, like other protein steps, is a double step characterized by 2 waves which follow each other in close succession (Fig. 1). When the height of these steps is plotted against concentration, a curve is obtained which has the shape of an adsorption isotherm. This fact agrees with polarographic theories. With decreasing concentration, the second part of the double step tends to decrease first, while the first part varies but little. On further dilution, the second part of the double step disappears completely and the first part develops a maximum, the relative height of which depends upon the dilution. Finally, in the greatest dilutions a cobalt maximum appears. This latter dilution is the minimum dilution at which, if the liquid is shaken, no foam appears.

This report deals primarily with the comparison of various samples of insulin. For the sake of comparison the measurements are based upon the height of the insulin double step of a 4.5 mg % solution of

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† For theory and application of polarography, see Heyrovsky,[4] and Brdicka.[1]

[4] Heyrovsky, J., Polarography in *Physikalische Methoden der analytischen Chemie*, Vol. II, Leipzig, 1936.

TABLE I.

Brand	A	B	C	D	E	F	G	H
Height of protein wave in mm	26	27	33	23	28	27	26	26

insulin prepared by the authors from crystals supplied by various manufacturers. Eight different brands were examined. Table I indicates the results.

Four samples of crystalline insulin in solution (commercial product) were obtained from manufacturers. All of the ampules were labeled 40 U per cc. These insulin solutions were then diluted 50 times. The average values of 10 measurements were the following:

Brand A	Lot 1: 25.5 mm	Lot 2: 25.5 mm
Brand B	Lot 1: 23.5 mm	Lot 2: 23.5 mm

There is no reason to doubt that the higher step corresponds to a higher concentration of available SS groups, as all single measurements were within ±1 mm of the average. These findings tend to show that different brands of crystalline insulin may have a different chemical composition or structure.

A comparison between solutions prepared from solid insulin and the commercial solutions of the same insulin brought out the following facts: The insulin concentration of the market product was determined by a polarographic comparison with the authors' solutions. From this concentration and the known activity of the sample (40 U per cc, according to the label), the activity of one mg of the solid insulin could be calculated. Table II gives the results of this experiment.

TABLE II.

	Brand A Crystalline	Brand B Crystalline	Brand A Amorphous
(I) Sample dissolved by the authors			
Concentration of solutions prepared by the authors	4.5 mg %	4.5 mg %	4.5 mg %
Height of polarographic step (Average of 5 readings)	25.5 mm	27 mm	18 mm
(II) Insulin solution, commercial, (40 U per cc), diluted 50 times			
Height of polarographic step (Average of 5 readings)	25.5 mm	23.5 mm	27.5 mm
Concentration of solid insulin calculated from height of polarographic step	4.5 mg %	3.9 mg %	6.9 mg %
Units per 100 cc (after diluting 50 times)	80	80	80
Units per mg of solid insulin	17.8	20.5	11.6

It is a well known fact that the activity of an insulin sample can be decreased or entirely destroyed by various physical and chemical agents. One of these is heat. A comparative analysis was made of 2 different lots of insulin before and after prolonged application of heat, which kept the temperature at 52°C for 10 days. In both instances, the activity of the sample dropped to about one-half of its original value; yet the polarographic behavior did not change at all. Fig. 2 illustrates this. One of the curves was obtained with the unheated sample labeled 40 U per cc. The other curve was obtained under the same conditions and represents the heated sample. According to the manufacturer the activity of this sample had dropped from 40 U per cc to 18.1 U per cc because of the application of heat. The polarographic curves obtained with these samples, so different in physiological activity, are identical. This tends to show that, so far, polarographic methods are inadequate as a basis for the determination of the physiological activity of an insulin sample.

From the work of Abel and Geiling[5] and du Vigneaud, *et al.*,[6] it appears that the reduction of SS to SH groups affects the activity of insulin, the SS groups thus being related to the active principle

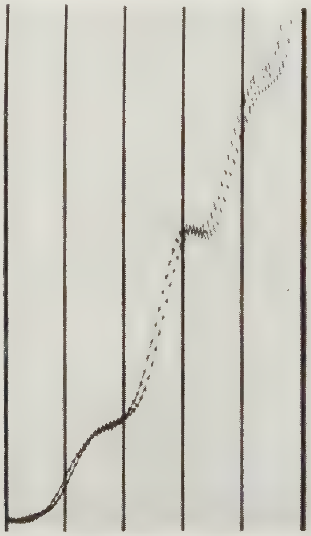

FIG. 2.

Curves of heated and unheated insulin sample. Written closely together for better comparison. First step indicates cobalt. Following double step indicates insulin protein. Galv. sens. 1/200.

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[5] Abel, J. J., and Geiling, E. M. K., *J. Pharm. and Exp. Ther.*, 1925, **25**, 423.

[6] du Vigneaud, V., *J. Biol. Chem.*, 1927, **75**, 393; 1931-32, **94**, 233.

of insulin. With the polarograph, SS as well as SH groups are registered. Based upon the findings of du Vigneaud, it could be assumed that insulin contains SS groups, but no free SH groups.

It is very unlikely that the heat treatment of the insulin samples changed about half of the SS groups to SH groups, and yet the findings do not contradict the assumption that the activity of insulin is related to its SS groups. Many substances contain SS groups without any physiological activity comparable to that of insulin. Inactivated insulin may be one of them. Therefore, it appears that if the SS groups are related to the activity, their presence constitutes but one of a series of conditions which must be fulfilled in order to obtain insulin activity.

Conclusions. 1. The height of polarographic steps of an insulin solution is a function of the concentration of SS (or SH) groups; it is independent of its intrinsic physiologic activity. 2. Polarographic analysis permits the determination of the concentration of insulin solutions, for a given brand, and after calibration.

10914

Lack of Carcinogenic Potency of Sulfanilamide and Prontosil Soluble in Mice.

Paul C. Zamecnik and Simon Koletsky. (Introduced by J. M. Hayman, Jr.)

From the Medical Service of Lakeside Hospital, and the Institute of Pathology, Western Reserve University, Cleveland, Ohio.

Because of the structural resemblance of sulfanilamide and Prontosil Soluble to o-amido-azotoluol,[1] B-naphthylamine,[2] "light green FS",[3] and other benzene derivatives which have been found to be carcinogenic,[4] the question of possible carcinogenic properties of these compounds naturally arises. Lewis reported on the lack ot carcinogenic potency of sulfanilamide in mice.[5] The present experiments were in progress at that time, and may be considered confirmatory evidence for Lewis's findings.

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[1] Shear, M. J., *Am. J. Cancer*, 1937, **29**, 269.

[2] Hueper, W. C., and Wolfe, H. D., Am. Assn. Pathol. Sci. Proc., *Am. J. Path.*, 1937, **13**, 656.

[3] Schiller, W., *Am. J. Cancer*, 1937, **31**, 486.

[4] Cook, J. W., and Kennaway, E. L., *Am. J. Cancer*, 1938, **33**, 50.

[5] Lewis, M. R., *Am. J. Cancer*, 1938, **34**, 431.

Ninety-seven male mice of the Bar Harbor dba strain, 2 to 3 months old, were used for the experiment. Fifty-two animals were given 40-60 mg injections of sulfanilamide at 2- to 3-week intervals over a 4½ month period, a total of 320 mg for each animal. A 20% finely ground olive oil suspension of sulfanilamide was used, so that a subcutaneous injection of 0.2 cc of suspension into the left axilla represented 40 mg. One mouse died during the injection period. At the end of 12 months, 23 animals were alive and were sacrificed. No gross evidence of tumor was found in any of these mice on postmortem examination, nor in the 27 which died of intercurrent disease over the preceding 6-month period. Microscopic examination of the organs of 10 of these animals showed no abnormal mitotic figures and nothing suggestive of possible malignant change in the liver, kidney, bone marrow, lung or bladder. Small cysts, containing clear fluid, were found near the sites of injection in 14 animals during the injection period, but these all later spontaneously disappeared.

Nineteen mice were injected with Prontosil Soluble,* over a 4½-month period—a total of 400 mg per animal. A warm 20% aqueous solution of Prontosil Soluble powder was prepared, of which each mouse received seven 0.2 or 0.3 cc subcutaneous injections into the left axilla, each injection containing 40 or 60 mg of Prontosil Soluble. Two mice died within 6 hours after injection, apparently of acute toxic effects produced by the Prontosil Soluble. At the end of 9 months, 12 animals were living. Eight mice survived 12 months, and were sacrificed. Postmortem examination showed no gross evidence of tumor in any of the animals. Microscopic examination of the organs of 5 of these mice likewise revealed nothing suggestive of malignant change in the liver, kidney, bone marrow, lung or bladder.

Of 25 control mice, 13 survived a year and showed no evidence of tumor. The mortality from intercurrent disease in the sulfanilamide group, the Prontosil Soluble group, and the control group over a year's time, was about the same.

Summary. Repeated subcutaneous injections of sulfanilamide and Prontosil Soluble in mice over a period of one year failed to produce tumors in these animals.

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* The Prontosil Soluble and sulfanilamide were furnished in powder form through the courtesy of the Winthrop Chemical Company. Prontosil Soluble is disodium 4′ sulphonamide benzene-2-azo-7-acetyl-amino-1-hydroxy-naphthalene-3-6-disulphonate.

10915

Dehydration and Basal Insulin Requirement.

D. B. Tyler, P. O. Greeley and D. R. Drury.
From the Department of Physiology, School of Medicine, University of Southern California.

It is a matter of common clinical experience in treating diabetic patients, that the amount of insulin required to produce a given effect may be quite different under different circumstances. It is often found that the insulin requirement for a patient on admittance to a hospital becomes much less as his condition becomes well controlled. This lack of effectiveness of insulin in diabetics not in good control, becomes very marked in coma when very large doses of insulin must be given to produce the effect that can be brought about by a small dose in the same patient when under good control.

In searching for a cause of this change in the effectiveness of insulin one thinks of the various accompaniments of uncontrolled diabetes such as glycosuria and ketosis. The most apparent result of glycosuria which tends to change the "internal environment" of the organism is dehydration. This factor was, therefore, the first variable chosen by us for investigation of its effect on insulin action and the particular insulin function we selected was the basal insulin requirement.* This latter may be defined as the rate at which insulin must be given to the fasting diabetic animal in order to maintain his blood sugar at a normal constant level. This requirement has been shown by Greeley[1] to be remarkably constant for an individual diabetic dog under standard conditions so that it would be easy to determine whether any desired factor could alter this requirement.

Dehydration can be readily produced in the diabetic by giving an excess of sugar or meat along with restricted water. We tried to do this without causing any other complication to supervene. This can be done by giving a dog a large amount of sugar or meat the day before the test and at the same time giving the usual insulin. The extent of dehydration was determined by changes in the water content of the plasma. For this, oxalated blood was collected and centrifuged in a well corked tube for 2 minutes. A weighed sample of the plasma was then dried at 105° to constant weight. For control figure we ran a series of plasma water content determinations on our diabetic dogs in good condition. We made 21 such determinations which gave an average water content of 93.0%—range 92.40 to 93.44%.

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* The insulin used in this work was contributed by the Eli Lilly Company.

[1] Greeley, P. O., *Am. J. Physiol.*, 1937, **120**, 345.

The determinations of the basal insulin requirement were carried out by the method described by Greeley.[1] This consists of first bringing the blood sugar to normal by a dose of 2 or 3 units of insulin and then determining the rate at which insulin must be injected to maintain the blood sugar at this normal level.

We determined first whether glycosuria without dehydration would affect the basal insulin requirement. For this we fed on the day before the run, a large excess of sugar with water *ad lib.* and the usual insulin. This was done on 3 dogs as follows:

Dog B_2 was given 300 g extra sucrose the day before the test with water *ad lib.* and usual insulin. Her urine volume for that day was 3304 cc with 87 g glucose, in contrast to a normal average of 600 cc and only a trace of sugar. The normal basal insulin rate had been previously established to be between 0.14 to 0.17 units per hour. The rate found during this run was 0.16 units per hour. That the dog was not dehydrated was apparent from the plasma water determination of 93.8 taken during the run.

Dog T_1, female, received 300 g of extra sucrose the preceding day with water *ad lib.* and insulin. This animal excreted 29.8 g of glucose and 2280 cc of urine in contrast to a normal of 500 cc and a trace of sugar. The normal basal insulin rate for this dog had been established at 0.08 to 0.11 units per hour. The rate found during this experiment was 0.09 units per hour with the plasma water 93.3%.

Dog C_1 received 475 g of glucose the preceding day with water *ad lib.* and usual insulin. This resulted in a urine volume of 1425 cc and 10.5 g of glucose. The rate found was 0.20 units per hour.

No basal rate was run on this last animal but the rate found can be compared directly with the rate required when the animal was dehydrated one week later. This was done by feeding extra glucose for 2 days prior to the experiment with the usual insulin. The animal lost 2 kg weight in these 2 days and the plasma water fell to 89.5%. The basal insulin rate was found to be 0.54 units per hour which is an increase of 170% over the rate found without dehydration.

Severe dehydrations were also produced in 2 other dogs. Dog B_1 was given 500 g extra sugar with 1000 cc water and the usual insulin the day before the test. It excreted 1650 cc urine with 41 g glucose. The basal insulin requirement was found to be .55 units per hour, which contrasted with the rate on this animal under standard conditions of .35 units per hour.

Dog H_1 was given 200 g extra sugar, restricted water, and 10 units insulin instead of the usual 18 units the day before the test. It lost 1 kg in weight, urine sugar and volume not recorded. The basal insulin requirement was found to be .60 units per hour which contrasts with the normal for this dog of .28 units per hour.

The foregoing are the most severe conditions of dehydration which we produced. Besides these we brought about moderate degrees of dehydration in our experimental dogs in order to see whether the less serious conditions, such as one might meet in ambulatory patients, would affect the basal insulin requirement. The dehydration was produced as in those cases already cited but the amount of the extra glucose or protein given was less; the amount of insulin given was not changed from the routine dosage administered on ordinary days to keep the animal in a "steady state". In other words, the dogs received the same dosage of insulin throughout the "dehydrating period" as they received every day, the only change being an increase in the "superfluous glucose". The results are given chronologically for each dog in Table I.

In these dogs there is seen to be a definite tendency of the basal insulin rate to increase with a reduction of the water content of the plasma.

TABLE I.

Date	Rate	Plasma Water	
Dog B_1, Female			
12-4-36	.41	91.90	Given extra sugar day before
12-23-36	.22	92.55	” ” ” ” ”
1-14-37	.45	91.18	” ” ” ” ”
1-20-37	.21	92.94	Normal
1-27-37	.15	92.23	”
2-4-37	.22	92.73	”
2-11-37	.16	93.80	”
2-16-37	.14	92.93	”
2-18-37	.20	90.66	Given extra sugar day before
2-23-37	.12	92.64	Normal
2-25-37	.24	91.70	Given extra meat day before
3-5-37	.42	91.49	” ” sugar ” ”
3-9-37	.17	92.40	Normal
3-18-37	.15	92.33	”
Dog T_1, Female			
1-27-37	.11	93.20	Normal
2-4-37	.12	92.66	”
2-11-37	.08	93.34	Given extra sucrose day before (no dehydration)
2-16-37	.08	93.24	Normal
2-17-37	.12	92.10	Slight dehydration
2-23-37	.12	93.05	Normal
2-25-37	.20	92.16	Given extra meat day before
3-5-37	.16	92.64	Extra sugar—no dehydration
3-11-37	.30	91.99	Given extra meat and sugar day before
3-18-37	.09	92.77	Normal

Our results do not necessarily conflict with those of authors who report that dehydrated animals show a prolonged action of insulin.[2] We measured only the effect of dehydration on the basal insulin requirement, the insulin given intravenously. All these authors cited gave the insulin subcutaneously; it is not improbable that dehydration slows up the absorption rate of insulin from the tissues. Others have shown that dehydration slows up the return of the blood sugar to normal after administration of glucose by stomach or intravenously.[3] Drabkin and Shilkret[4] report that spastic convulsions were not observed in desiccated animals after insulin although they appeared in those given fluids.

Summary. Depancreatized dogs maintained on a standard regime were dehydrated by producing glycosuria along with restricted water intake. It was found that the basal insulin requirement of the animals in this state was increased above that which they had normally without dehydration, but with other conditions the same.

If the dogs be given enough water so that no dehydration results from the glycosuria no increase in the basal insulin requirement results.

10916

Choline Esterase and Esters of Thiamine.

DAVID GLICK AND WILLIAM ANTOPOL.
From the Laboratories of the Newark Beth Israel Hospital, Newark, N. J.

The possible relationship between the physiological rôles of thiamine and choline esterase led the authors, in a previous study,[1] to investigate the inhibition of the enzyme by thiamine. Since thiamine occurs in the animal body largely as the pyrophosphate ester, cocarboxylase, a study of the effect of this compound upon choline esterase was undertaken, and it was found that, in contrast to the pronounced inhibitory action of thiamine, the pyrophosphate ester produced very little inhibition.

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[2] Andrews, E., *Arch. Int. Med.*, 1926, **38**, 136; 1927, **40**, 637.

[3] Tisdale, F. F., Drake, T. H., and Brown, A., *Am. J. Dis. Child.*, 1925, **30**, 837; *ibid.*, 1926, **32**, 854.

[4] Drabkin and Shilkret, *Am. J. Physiol.*, 1927, **83**, 141.

[1] Glick, D., and Antopol, W., *J. Pharm. and Exp. Therap.*, 1939, **65**, 389.

It has been reported[2] that although ethers are inactive, the esters of thiamine are physiologically active upon polyneuritic animals. There is the possibility that the ester is hydrolyzed *in vivo,* and the effects observed are due in fact to liberated thiamine. An attempt to elucidate this point was made by an investigation of both the enzymatic and hydroxyl-ion hydrolysis of the acetyl ester of thiamine chloride. A slow enzymatic splitting at a pH of 7.4, accompanied by a non-enzymatic scission of practically the same magnitude, was observed. Hence it cannot be stated with certainty that the physiological activity of the ester is an intrinsic property of the compound itself. However, the probability that such is the case gains credence by analogy to the physiological effects of somewhat related esters such as acetylcholine, atropine, and cocaine. As is well known, esterification enhances the physiological response to many substances. The inhibition of choline esterase by acetylthiamine chloride was also studied, the conditions employed being such as to obviate the disturbing influence of hydrolysis of the inhibitor.

Kuhn, Wieland, and Huebschmann[3] have raised the question of chemical mediation of nerve impulses by acetylthiamine. One would expect that the mediator would be capable of rapid removal from the site of its action. The relatively slow rate of hydrolysis, both enzymatically and non-enzymatically, reported below does not speak in favor of this rôle for acetylthiamine.

The pure thiamine chloride pyrophosphate and the acetyl ester of thiamine chloride were kindly supplied by Dr. R. T. Major of the Merck Research Laboratory, Rahway, N. J.

Measurements of ester hydrolysis were carried out in essentially the same manner as previously by the manometric method employing the Warburg apparatus.[1] All of the experiments were conducted at a pH of 7.4 and 30°. For the inhibition investigations 3 ml of acetylcholine chloride solution (5 mg of ester per ml in bicarbonate-Ringer solution) were employed with 0.5 ml of inhibitor solution and 0.5 ml of enzyme solution (0.4 ml of horse serum diluted to 10 ml with bicarbonate-Ringer solution). The inhibitor solutions were prepared by adding 0.1 n NaOH to the weighed substances until neutralized to pH 7.4, and the appropriate dilutions were then made with bicarbonate-Ringer solution. Readings were taken at 5-minute intervals for 30 minutes and the slopes of the linear activity-time curves were used to compare the effects of various concentrations of the inhibitors. The data obtained are shown in Fig. 1. The curve for the inhibition by thiamine has been taken from a previous publication[1] and was included to enable direct comparison with the inhibitory actions of the esters. Data in Fig. 1 were used to construct the curves in Fig. 2, which in turn were used to calculate the affinity of the inhibitors for the enzyme in the same manner as previously.[1] The calculation follows that given by Ziff, Jahn, and Renshaw.[4] The ratio of the slopes of the two lines to their respective intercepts on the 1/K axis is equal to their affinities for choline esterase relative to that of acetylcholine taken as 1. Hence, the affinity of the pyrophosphate ester is 1143/690 or 1.7, while that of the acetyl ester is 3500/665 or 5.3, as compared to 26 found previously[1] for the free thiamine.

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[2] Cline, J. K., unpublished data quoted by Williams, R. R., and Speis, T. D., in *Vitamin B_1 and Its Use in Medicine,* p. 173, The Macmillan Co., New York, 1938.

[3] Kuhn, R., Wieland, T., and Huebschmann, H., *Z. Physiol. Chem.,* 1939, **259**, 48.

For the study of the hydrolysis of acetylthiamine 1.5 ml of ester solution (1⅓%) and 0.5 ml of enzyme solution (0.5 ml horse serum diluted to 10 ml) were employed. The quantity of free thiamine liberated by hydrolysis had no demonstrable effect upon the course of the enzymatic reaction since the linearity of the activity-

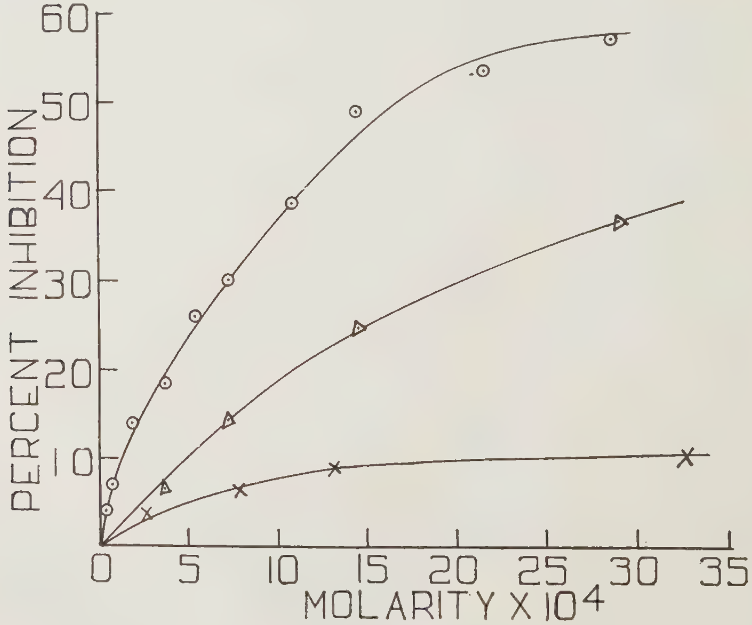

FIG. 1.

Inhibitions of choline esterase by thiamine (⊙), thiamine pyrophosphate (×), and acetylthiamine (△).

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[4] Ziff, M., Jahn, F. P., and Renshaw, R. R., *J. Am. Chem. Soc.*, 1938, **60**, 178.

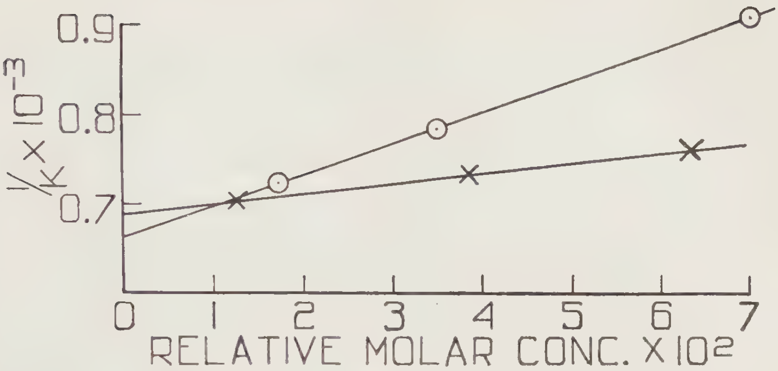

FIG. 2.

Inhibition of choline esterase by thiamine pyrophosphate (×) and acetylthiamine (⊙) in relation to the acetylcholine concentration. K, monomolecular velocity constant ($^{2.3}/_{t} \log {^{a}/_{a-x}}$). Abscissæ, ratio of molar concentration of thiamine ester to that of acetylcholine.

time curve was maintained for the duration of the experiment, 300 minutes. Furthermore, maximum velocity of enzymatic hydrolysis was obtained by employing a sufficiently high substrate concentration. The purely enzymatic hydrolysis was equivalent to the liberation of 26 cmm CO_2 in 300 minutes, and the scission due to hydroxyl-ion in the same period was 22 cmm.

After this paper had been submitted for publication, two others appeared concerning the enzymatic hydrolysis of acetylthiamine. Süllmann and Birkhäuser[5] reported that brain extract was able to split the ester, though horse serum had practically no effect. Their failure to observe hydrolysis in the latter case was due, for the most part, to the fact that measurements, under the conditions employed, were made for too short a reaction period (60 minutes). These authors also observed the inhibition of choline esterase by thiamine and acetylthiamine. Massart and Dufait[6] found that horse serum hydrolyzes acetylthiamine, and that eserine inhibits the action.

Summary. The acetyl ester of thiamine chloride was found to be a poorer inhibitor of choline esterase than free thiamine, while the pyrophosphoric ester had even less inhibitory action. The affinity for the enzyme of the former was calculated to be 5.3, and of the latter 1.7, times that of acetylcholine. The acetyl ester was hydrolyzed slowly by horse serum, the magnitude of the enzymatic splitting being approximately equal to that due to the hydroxyl-ion scission at the same pH (7.4).

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[5] Süllmann, H., and Birkhäuser, H., *Schweiz. Med. Woch.*, 1939, **69**, 648.

[6] Massart, L., and Dufait, R., *Naturwissenschaften*, 1939, **27**, 567.

10917

Influence of Hypochloremia upon the Convulsive Reactivity.*

E. Spiegel and H. Wycis.

From the Department of Experimental Neurology, D. J. McCarthy Foundation, Temple University, School of Medicine, Philadelphia, Pa.

Our knowledge of the influence of the chloride content of the blood upon the convulsive reactivity is rather contradictory.

Lennox and Cobb[1] mention findings indicating that increased concentration of chloride in nervous tissues may play a contributory part in seizures. In their tentative list of conditions which may precipitate seizures they mention high chloride, and among the conditions which may prevent seizures, low chloride. These statements were widely accepted, *e. g.*, by Wolff.[2] On the other hand, the convulsive reactivity may be increased in conditions associated with loss of chlorides such as prolonged vomiting in children (Gordon[3]). In view of the practical importance of the question whether the production of hypochloremia in patients with convulsive disorders is desirable or not, in the following series of experiments the influence of hypochloremia upon the convulsive reactivity was quantitatively studied.

The experiments were performed on 8 rabbits and 2 cats. The convulsive reactivity of the animals was determined by electrical stimulation through the eyeballs using an apparatus devised by one of us (Spiegel[4]), that permits one to vary the voltage as well as the duration of the alternating current. After a relative constancy of the threshold was established in daily measurements, hypochloremia was produced by intraperitoneal injection of 10% glucose solution (50 cc per kilo body weight, in some cases repeated after 4 hours following the procedure of Silvette and Britton[5]). Six hours after the first injection the convulsive reactivity was again determined. The chloride content of the serum was determined by the Myers-Whitehorn[6] method before and 6 hours after the glucose injection

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* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Lennox, W. G., and Cobb, St., *Epilepsy, Medicine*, 1928, **7**, 105.

[2] Wolff, H. G., *J. Am. Med. Assn.*, 1939, **112**, 1250.

[3] Gordon, H. H., *J. Am. Med. Assn.*, 1939, **112**, 1252.

[4] Spiegel, E., *J. Lab. and Clin. Med.*, 1937, **22**, 1274.

[5] Silvette, H., and Britton, S. W., *Am. J. Physiol.*, 1935, **111**, 305.

[6] Myers, V. C., *J. Lab. and Clin. Med.*, 1920, **6**, 17; Whitehorn, J. C., *J. Biol. Chem.*, 1921, **45**, 449.

TABLE I.

Animal No.	Convulsion threshold				Duration of convulsions		Serum Cl (mg/100 cc)	
	Before hypochloremia		During hypochloremia		Before hypochloremia sec	During hypochloremia sec	Before injection	After injection 10% glucose, 50 cc/kg intraperitoneally
	Range	Avg A	H	*H/A				
	Milliampère seconds							
Rabbit								
20	12-16	13.6	17.5	1.3	15-60	33	371	349
36	12-16	14.0	14	1.0	11-30	19	337	313
			7	0.5		50		235‡
45	12-17.5	14.0	14	1.0	10-52	10	371	365
			5†	0.36		64		127‡
49	12-14	13.1	7†	0.53	13-32	20	355	217‡
78	19-21	20.6	16	0.77	24-26	16	327	279
81	10.5-16	12.6	14	1.1	12-57	18	325	301
			7	0.55		24		265
702	9-10.5	9.3	21	2.2	24-40	17	343	271
703	12-14	12.8	14	1.09	13-50	34	301	292
Cat								
38	17.5-26	22.2	17.5	0.78	45-94	60	374	205
64	24.5	24.5	19	0.77	34-80	146	343	283

* H: convulsion threshold in hypochloremia; A: average convulsion threshold before hypochloremia.

† The threshold returned some days later to 19 in rabbit No. 45, and to 17.5 in rabbit No. 49

‡ Serum Cl returned some days later to 343 in rabbit 36, to 331 in rabbit 45, to 352 in rabbit 49.

(immediately before the determination of the convulsion threshold).

The results are summarized in Table I in which the convulsion threshold is expressed in milliampère-seconds. In all experiments in which the chloride value of the serum dropped below 265 mg per 100 cc the convulsion threshold was lowered, the ratio of the threshold in hypochloremia to the mean threshold before the induction of the hypochloremia (H/A) dropping below 1. In 2 cases (rabbit No. 78, cat No. 64) a decrease of Cl to 279 mg and 283 mg respectively sufficed to lower the convulsive reactivity; only in rabbit No. 702 the threshold was increased while the Cl was lowered to 271 mg. In this animal, however, the duration of the convulsions was decreased. When the values of the Cl were not lowered below 301 (first experiment in rabbits 36, 45, and 81, rabbit 20) also the threshold was not diminished. The lowest value of H/A was observed in the animal (rabbit 45) with the most marked hypochloremia (127 mg Cl). There is, however, no definite parallelism between degree of hypochloremia and decrease of the convulsion threshold; *e. g.*, in cats No. 38 and 64, the ratio H/A had nearly the same value, while the hypochloremia was more marked in cat 38 than in cat 64. In 3 experiments the increased convulsive reactivity in hypochloremia was also manifested by an increase of the duration of the convulsions, (No. 36, 45, cat 64); in 3 cases the duration of the convulsions elicited during hypochloremia was within the range observed before the hypochloremia (rabbit 49, 81, cat 38) and in one animal it was diminished (No. 78).

As pointed out by Silvette and Britton, intraperitoneal glucose injection lowers both Na and Cl, the loss of these 2 ions showing a straight line relationship. In experiments of Fenn,[7] *et al.*, nervous tissue immersed in isotonic sugar solution lost Na and Cl in approximately equal amounts. The loss of salt from the tissues results in a shift of extracellular water into the cells producing swelling of the cells as was shown on the red blood corpuscles by Gilman.[8] Swelling of the brain, as for instance induced by injection of hypotonic solutions, is associated with increase of the permeability of the cellular surface films for ions (Spiegel and Spiegel-Adolf[9]) thus facilitating those mechanisms upon which excitation depends.

The above mentioned experience that hyperchloremia also may increase the convulsive reactivity is not in contradiction to the results of the present series of experiments. As was pointed out by Spiegel and Spiegel-Adolf, the convulsive reactivity may be increased by 2 mechanisms, pericellular increase of the concentration of ions on the one hand, increase of the permeability of the cellular surface films on the other hand. While the effect of hypochloremia belongs to the second group, hyperchloremia probably acts according to the first mentioned mechanism.

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[7] Fenn, W. O., Cobb, D. M., Hegnauer, A. H., and Marsh, B. S., *Am. J. Physiol.*, 1934, **110**, 74.

[8] Gilman, A., *Am. J. Physiol.*, 1934, **108**, 662.

[9] Spiegel, E., and Spiegel-Adolf, M., *Am. J. Psychiat.*, 1936, **92**, 1145; PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 799.

Summary. Experiments on rabbits and cats showed that hypochloremia increases the convulsive reactivity

10918

Negative Effects of Cysteine Hydrochloride on Regression of Carcinoma in Line A Albino Mice.

E. Elizabeth Jones. (Introduced by George Van S. Smith.)

From the Fearing Research Laboratory, Free Hospital for Women, Brookline, Mass. (the Reginald W. Bird Endowment), and Wellesley College, Wellesley, Mass.

Cysteine hydrochloride injected directly into the Jensen rat sarcoma has been reported to produce complete regression of the tumor.[1] Recurrence did not occur within a period of 6 months, and reinoculation with the same tumor or with Emge sarcoma[2] was not possible after regression of a cysteine treated tumor. Acids adjusted to the same pH as the cysteine hydrochloride solutions and injected in equivalent volumes were reported to be rapidly absorbed from the tumor and, except for a small area of necrosis, to produce no cytological change.[3]

After the experiments here described had been completed, further work was reported[4] on the treatment of 3 different rat tumors, and on the Brown-Pearce rabbit carcinoma. This confirmed the earlier observations that direct injection of cysteine hydrochloride into Jensen's rat sarcoma and an adenofibroma of rats caused complete regression and cross immunity between the two. Treatment administered by other route was practically ineffective except that it prolonged the average life of rats bearing the Walker tumor by 11.1 days.

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[1] Connor, C. L., Carr, J. L., and Ginzton, L., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 374.

[2] Carr, J. L., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 341.

[3] Carr, J. L., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 343.

[4] Carr, J. L., Connor, C. L., and Ginzton, L. L., *Am. J. Cancer*, 1936, **31**, 428.

Experiments with cysteine hydrochloride were carried on in this laboratory over a period of several months. Spontaneous and inoculated carcinomata in Line A albino mice were used. Thirteen spontaneous mammary gland tumors were directly injected from 1 to 6 times at intervals of 4 to 6 days. Ten mice received 15 mg cysteine hydrochloride in 0.15 cc distilled water at each injection and 3 were similarly treated with 10 mg in 0.1 cc. Central necrosis and extrusion of necrotic tissue occurred in 10 of these, as described by Carr. In 3 cases no extrusion of material occurred, and the tumors appeared to be only slightly affected by the treatment. In these animals death followed within 3 to 4 weeks with no evidence of regression. Of the 10 tumors in which necrosis was present: 3 regressed completely, but the mice died within 1 week after the first injection; 1 regressed completely, with no recurrence at the time of death 45 days later; 5 regressed partially, but the outer shell of tumor tissue which remained continued to grow, as evidenced by the presence of mitotic figures. In 1 mouse with 2 tumors, injections were made into 1 tumor only; this tumor regressed but reappeared after 3 weeks. During this period the second tumor continued to grow. After recurrence of the first tumor, the second tumor was injected and partial regression of this tumor took place without influencing the growth of the first tumor.

Since the action of the cysteine hydrochloride seemed to resemble so closely that of a caustic substance, injections of hydrochloric acid of the same pH and in equivalent volumes were tried and found to produce the same effect as cysteine hydrochloride. Following these tests, cysteine hydrochloride solutions were neutralized by the addition of NaOH, and 4 mice with spontaneous tumors were injected with this solution. In only one of these was there any marked regression, but mitotic figures were found in the outer areas of the tumor in sections taken at autopsy on the nineteenth day after treatment. None of these mice survived a period of 6 weeks.

Inoculated carcinomata in 19 mice behaved in a similar manner following injections with cysteine hydrochloride and with neutralized solutions. Central necrosis occurred in many but the edges or base of the tumor continued to grow. In a series of 20 mice doubly inoculated with adenocarcinoma 478, injections of cysteine hydrochloride were given into one tumor only. The treated tumors became necrotic and large portions of them were sloughed off, but no complete regressions occurred and growth continued at the base and edges. The growth of the untreated tumor was not affected by the necrosis of the tumor on the opposite side.

Summary. Cysteine hydrochloride injected directly into spontaneous and inoculated carcinoma caused necrosis and extrusion of necrotic material but produced permanent regression in only one animal. Partial regression of one tumor was without effect on the growth of a second untreated tumor.

10919

Some Effects of Anesthetic Mixtures Dissolved in Oil on Motor Nerves in the Cat.

Donald Duncan. (Introduced by G. M. Decherd, Jr.)
From the Department of Anatomy, University of Texas, Galveston.

Since the introduction of local anesthetic substances dissolved in oil by Yeomans, Gorsch and Mathesheimer,[1] mixtures patterned after the original have been extensively used by proctologists.[2] Recently the use of these substances has spread to other clinical specialties.[3, 4] Injections of the various mixtures regularly produce anesthesia lasting from several days to as much as one month, and occasionally longer.[5] Variable amounts of muscular relaxation for similar periods are also obtained.

The usual explanation for the prolonged effect is gradual release of the anesthetic materials from the oily vehicle whereby a long continued depressant effect on the functional activity of the nerve endings is obtained.[1, 6] However, on the basis of Donaggio's[7] work with long continued application of aqueous local anesthetic solutions, one might expect irreversible organic changes leading to nerve degeneration. Degeneration as a result of the injection of local anesthetics in oil has been suggested in only one[8] of the many clinical papers examined, and apparently no animal experiments followed by histological examination of the nerves have been performed.

In this laboratory cats were injected in an attempt to determine the effects of anesthetics in oil on the peripheral motor nerves. For this purpose 3 preparations were tried. The first was a locally prepared mixture containing procaine base 1.5, butesin 6.0, benzyl alcohol 5.0 and sterilized oil of sweet almonds, U.S.P., q.s. 100.0. The other 2 were proprietary mixtures supplied by the manufacturers.* One contained nupercaine hydrochloride (alpha-butyloxy-cinchoninic acid-diethyl-ethylene-diamide hydrochloride) 0.5, benzyl alcohol 10.0, phenol 1.0, and sterilized oil of sweet almonds q.s. 100.0. The last mixture tried contained eucupine base (isoamylhydrocuprein) 0.1, ethylaminobenzoate 3.0, benzyl alcohol 5.0, and sterilized oil of sweet almonds q.s. 100.0.

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[1] Yeomans, F. C., Gorsch, R. V., and Mathesheimer, J. L., *M. J. and Rec.*, 1928, **127**, 19.

[2] Green, W. W., *Tr. Am. Proct. Soc.*, 1937, **38**, 153.

[3] Hollander, E., *Arch. Neurol. and Psychiat.*, 1938, **40**, 743.

[4] Hunter, G. W., *Am. J. Obst. and Gynec.*, 1939, **38**, 318.

[5] Block, L. H., Greene, B. L., and Wiltrakis, G. A., *Illinois M. J.*, 1938, **73**, 238.

[6] Manheim, S. D., and Marks, M. M., *Am. J. Surg.*, 1938, **39**, 86.

[7] Donaggio, A., *Gior. ital. di anest. e di analg.*, 1937, **3**, 325.

[8] Steinberg, N., *New England J. Med.*, 1936, **215**, 1019.

Initial experiments with the procaine and nupercaine mixtures were directed towards the femoral and sciatic nerves. Neither solution produced a complete block of the motor fibers when as much as 5 cc was injected around the trunks of these nerves. Various degrees of weakness lasting for hours or days were obtained, but after 10 trials including exposure of the nerves and direct injection into their sheaths the attempts were abandoned.

The subcutaneous tissues of the face were then infiltrated to test the effect on branches of the facial nerve. This method had the advantage of acting on the nerve supply of a muscle, the *orbicularis oculi,* which is easily tested for paralysis. Injections were made with a 22 gauge needle and the material was introduced slowly and in such a manner as to obtain wide distribution of the oil and also to prevent large local accumulations. The injections were done under light ether narcosis and the amounts varied between 3 and 5 cc depending on the size of the animal.

Four trials each with the procaine and nupercaine mixtures produced an immediate complete paralysis of the *orbicularis oculi* in every case. Complete paralysis as evidenced by absence of movement of the lids on corneal stimulation lasted from 3 hours to 3 days in the case of the procaine mixture and from 2-21 days with the nupercaine mixture. Partial paralysis indicated by incomplete closure of the lids and weakness on forcible opening lasted several additional days. Four trials with the eucupin mixture, 5 cc in each case, did not produce evidence of paraysis for more than one hour. A fifth 5 cc injection caused a paralysis which lasted for 12 days. Six injections of 5 cc of sterilized almond oil were not followed by paralysis nor by any discernible evidence of weakness.

Ten to 30 days following the injections, the cats were killed with chloroform and the facial nerve and its branches were carefully dissected out and placed in 1% osmic acid for 24 hours. Portions of each specimen were then teased in glycerine and examined. In the 3 instances of complete paralysis of the *orbicularis oculi* lasting 14 days or longer, degeneration of the fibers to the muscle was complete. The histological picture was one of the typical Wallerian degeneration such as follows the traumatic section of a nerve. In 2 of these animals 30 days had elapsed and the muscle was again active. In these cases regenerating fibers were numerous. In all of the other animals injected with an active solution varying numbers of degenerating fibers were seen in the branches of the facial nerve on the injected side. In those receiving eucupin without effect the abnormal fibers were not numerous but were well above the number that might be expected to occur in a normal animal.[9] With the other 2 preparations the number of degenerating fibers was greater and in general corresponded to the duration of the paralytic effect of the injection.

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* Nupercaine, Ciba Pharmaceutical Products, Inc., Summit, N. J. Eucupin, Rare Chemicals, Inc., Nepara Park, N. Y.

The state of degeneration indicated that the fibers so affected were killed almost immediately; *i. e.,* in no case was there seen a mixture of early and late degenerative changes suggestive of a prolonged functional depression followed by physical disintegration.

No more than a very occasional fiber exhibiting degeneration was seen following the injection of almond oil alone. Most of the branches from specimens so treated were composed exclusively of intact fibers.

The conclusion reached is that oil solutions of the local anesthetics owe their prolonged action, at least in part, to degeneration of nerve fibers in the injected area. Complete motor block of more than 4 days is accompanied by complete degeneration of the motor nerves to the paralyzed muscle. Subsequent restoration of function is due to regeneration.

Summary. Mixtures of local anesthetic substances dissolved in oil were injected into the subcutaneous tissues of the face in cats. Paralysis of the *orbicularis oculi* muscle was invariably obtained with solutions containing either procaine base or nupercaine hydrochloride. The paralysis lasted from several hours as a minimum to a maximum of 21 days. Histological examination of the tissues 10-31 days after injection revealed degeneration of facial nerve fibers in every case. The amount of degeneration varied from a few scattered fibers to complete in some branches. In general the amount of degeneration corresponded to the duration of the paralysis. Injections of the oil vehicle alone caused no paralysis and no degeneration.

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[9] Duncan, D., *J. Comp. Neurol.*, 1930, **51**, 197.

10920

Effect of Sulfapyridine in Pneumococcus Type I Infections In Rabbits.

W. Paul Havens, L. P. Hansen and Cecelia G. Kramer.
(Introduced by Hobart A. Reimann.)

From the Departments of Medicine and Physiological Chemistry, Jefferson Medical College and Hospital, Philadelphia, Pa.

Since much of the experimental work with sulfapyridine has been done with mice, it was decided to test the chemotherapeutic action of this drug against bacteremia with Type I pneumococcus in rabbits.

According to the method of Goodner[1] 0.1 cc of a 1:1000 dilution of an 18-hour blood broth culture of Type I pneumococcus was inoculated endermally into the shaved skin of 40 rabbits. Twenty were kept as controls and the remaining 20 were given sulfapyridine at intervals ranging from 12 to 24 hours following inoculation. Blood cultures were made just before the onset of treatment and daily thereafter. A record was kept of the number of colonies of pneumococci per cubic centimeter of blood each day. The drug was suspended in 60 to 80 cc of physiologic salt solution and administered by stomach tube 2 or 4 times a day at 4-hour intervals. The amount of sulfapyridine in the blood, determined by the method of Marshall and Litchfield,[2] was measured just before each treatment in every animal. Rectal temperatures were recorded twice daily. In every instance bacteremia was present before the onset of therapy.

As a preliminary step to determine the rate of absorption and excretion of the drug 2 uninfected rabbits were given 0.25 g of sulfapyridine per kilo of body weight in 2 equally divided doses 4 hours apart. While the maximum concentration appeared in the blood one and one-half hours after administration of each dose, a level almost as high was present one-half hour following treatment. Although the ability to conjugate the drug varied in different rabbits, the maximum amount of conjugation occurred in the first half hour. Twenty hours after treatment only a trace of the drug in its conjugated form was left in the blood.

Group I. Three pairs of infected animals were treated according to the above program, 16, 19 and 21 hours respectively following inoculation. Bacteremia was present and the infection was apparently overwhelming. One treated and one control animal of the series treated 16 hours after infection survived. The surviving treated animal maintained a level of 6 to 10 mg of total sulfapyridine in its blood, largely in the free state for approximately 8 hours a day, which dropped back to a low level at the end of each 24-hour period.

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[1] Goodner, K., *J. Exp. Med.*, 1928, **48**, 413.

[2] Marshall, E. K., Jr., and Litchfield, J. T., Jr., *Science*, 1938, **88**, 85.

Group II. Because of the inefficiency of therapy as just described when the concentration of the drug in the blood was allowed to drop over a period of 16 hours, another group of rabbits was treated 12 to 24 hours following infection with larger quantities of sulfapyridine given in 4 equal doses at 4-hour intervals, allowing only 8 hours during which an effective level might not be maintained. An attempt was made to obtain a high level in the blood at once by giving 0.75 g per kilo per day for the first day followed by 0.50 g per kilo thereafter. By this method the level in the blood rose 2 to 3 mg higher during the first 8 hours than when 0.5 g per kilo per day was given from the beginning of treatment. Of the 24 animals observed in this group 4 of the treated (33.3%) died while 8 of the controls (66⅔%) died.

Group III. Two controls and 2 infected rabbits treated with 0.75 g of sulfapyridine per kilo per day were observed. Both treated animals maintained levels of 11 to 15 mg in the blood over several hours of the day for 5 days. Blood cultures became sterile but the concentration of sulfapyridine rose in the blood to as high as 30 mg, including 10 to 12 mg of the conjugated form. The animals became weak, emaciated and one developed urinary retention. Both of the treated and one of the control animals died.

Comment. There is some benefit to be ascribed to treating pneumococcus Type I bacteremia in rabbits with sulfapyridine if a level of 7 to 10 mg of total sulfapyridine is maintained for most of the 24-hour period of the day. To do this most effectively 0.75 g per kilo per day in 4 equal doses at 4-hour intervals should be given the first day followed by 0.5 g per kilo per day thereafter until blood cultures are negative for 3 days. The proportion of free and conjugated drug is apparently of little importance in rabbits in relation to bacteriostatic efficiency. However, in those animals believed to manifest toxic symptoms from high concentrations of total sulfapyridine (20 to 30 mg) it was observed that the conjugated drug was often as high as 10 to 14 mg. The animals which recovered were able to utilize and eliminate the drug and showed a progressive diminution in the number of pneumococci in the blood over a period of 3 to 5 days when the blood culture ordinarily became sterile. With very high concentrations of sulfapyridine, bacteremia at times persisted, suggesting the possibility that the high levels of the drug are so toxic, or that the amount of polysaccharide capsular substance is so large that the animal is overwhelmed and cannot utilize the normal protective mechanism which might be sufficient ordinarily to control the infection. The temperatures of the treated animals were usually 1 to 2 degrees F lower than the controls. The skin lesions of the treated animals are ordinarily less extensive, while the controls frequently presented more edema, swelling and necrotic areas.

Conclusion. 1. A blood level of 7 to 10 mg total sulfapyridine is effective in controlling bacteremia with Type I pneumococcus in 66.6% of treated rabbits. 2. In animals treated with sulfapyridine the local skin lesions are usually less extensive and the temperatures are 1 to 2 degrees lower than the controls.

10921

Observations on the Toxicology of Sulfathiazole and Sulfapyridine.

H. B. VAN DYKE, R. O. GREEP, GEOFFREY RAKE AND C. M. McKEE.

From the Squibb Institute for Medical Research, New Brunswick, N. J.

Fosbinder and Walter as well as Lott and Bergeim[1] have prepared the thiazole analogue of sulfapyridine, 2(*p*-amino-benzene-sulfonamido)thiazole, which in this and the following report will be called sulfathiazole. The pharmacology of this drug is of interest because it resembles sulfapyridine in respect of its chemotherapeutic efficacy in combating pneumococcus and other infections in mice.[2]

Experiments which have been performed to determine the acute lethal effects of a single dose of the sodium salt of either drug are listed in Table I.* Each experiment represents observations in a single group of albino mice homogeneous as to breed and size. In experiments B, C, D, and E, the toxicity of the sodium salts of sulfapyridine and sulfathiazole after subcutaneous injection was compared at the same dose levels. In experiment B, the drugs appeared to be equally toxic; however, in experiments C, D, and E, when larger doses were administered, sulfapyridine sodium was much the more toxic. In experiment F, both drugs were administered by stomach tube. In this experiment, as in the others, sulfapyridine sodium appears to be the more toxic. "Characteristic" curves drawn from the data of experiments A-E indicate that the LD50 of sulfapyridine Na is about 0.95 g per kilo, whereas that of sulfathiazole Na is about 1.45 g per kilo. The determination of the toxicity of a single dose of the free acids was not attempted because their solubility is low and their absorption is probably extremely variable.[3]

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[1] Fosbinder, R. J., and Walter, L. A., *J. Am. Chem. Soc.*, 1939, **61**, 2032; Lott, W. A., and Bergeim, F. H., in press.

[2] McKee, C. M., Rake, Geoffrey, Greep, R. O., and van Dyke, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 417.

* The molecular weight of sulfathiazole is 2.5% greater than sulfapyridine. Usually doses were corrected for this difference.

The effects of repeated doses of the free acids were, however, investigated in mice, rats, and monkeys. Mice were fed the Sherman No. 1 diet containing the drugs in concentrations of 1 and 2%. The technics of Bieter and coworkers[4] and Litchfield and coworkers[5] were employed to insure accurate estimation of the amounts of food and drug eaten. The data, summarized in Table II, indicate that

TABLE I.
Toxicity of Sodium Salts of Sulfapyridine and Sulfathiazole Administered as a Single Dose to Mice.

Experiment	Drug	Route of administration	Dose, g/kg	No. of mice	Mortality, %	Time of observation, hr
A	Sulfathiazole Na	Subcut.	0.5	25	4	45
	" "	"	1.0	25	20	45
	" "	"	2.0	25	92	45
	" "	"	3.5	25	100	6
	Alkali control	"	—	25	0	45
B	Sulfapyridine Na	"	0.500	23	4	72
	Sulfathiazole Na	"	0.512	22	4	72
C	Sulfapyridine Na	"	0.750	30	17	72
	Sulfathiazole Na	"	0.768	30	3	72
D	Sulfapyridine Na	"	1.000	30	98	72
	Sulfathiazole Na	"	1.025	30	13	72
E	Sulfapyridine Na	"	1.000	30	100	4
	Sulfathiazole Na	"	1.025	30	17	72
F	Sulfapyridine Na	Stomach tube	1.000	16	19	24
	" "	" "	1.500	16	25	24
	" "	" "	2.000	16	31	24
	Sulfathiazole Na	" "	1.025	15	0	24
	" "	" "	1.537	15	0	24
	" "	" "	2.050	16	6	24

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[3] Marshall, E. K., Jr., Bratton, A. C., and Litchfield, J. T., *Science*, 1938, **88**, 597.

[4] Bieter, R. N., Larson, W. P., Cranston, E. M., and Levine, M., *J. Pharm. and Exp. Ther.*, 1939, **66**, 3.

[5] Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *J. Pharm. and Exp. Ther.*, 1939, **66**, 23.

when the drugs are repeatedly consumed by mice sulfathiazole is distinctly more toxic than sulfapyridine if the concentration of the drug fed is 2%. No greater toxic effect of sulfathiazole could be detected in mice eating diets containing 1% of either drug. Groups of growing albino rats were fed diets containing 0.5 and 1% of the drugs for a period of 57 days. The weight-curves of the female animals are shown in Fig. 1 and demonstrate that in the rat sulfapyridine is the more toxic drug.

TABLE II.
Effects of Repeated Doses of Sulfapyridine and Sulfathiazole on Mice.

Drug	Amount in food, %	Days on diet	Mean wt		No. of survivors on day							Mean total dose of drug eaten by survivors, g
			at start, g	at end, g	0	5	10	15	20	25	28	
Sulfapyridine	1	28	12.06	19.74	14	14	14	14	14	14	14	0.814
”	2	28	15.55	18.56	28	28	28	28	27	27	27	1.477
Sulfathiazole	1	28	14.23	19.49	15	15	15	15	15	15	15	0.908
”	2	28	15.11	17.5	30	26	18	11	9	7	6	1.523

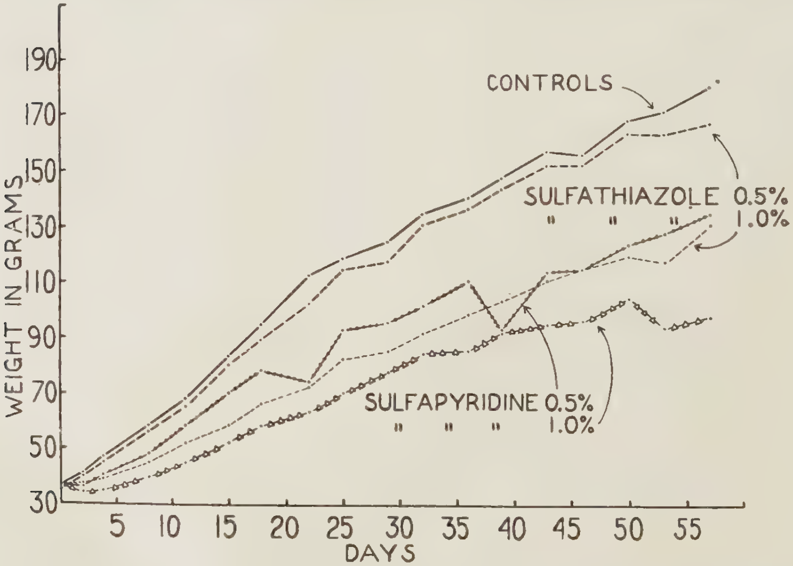

FIG. 1.

Average growth of homogeneous groups of 9 female rats receiving sulfathiazole or sulfapyridine in the food. No deaths occurred.

Comparable results were obtained in a similar experiment with growing males. However, during the experimental period, a sudden severe unexplained loss of weight occurred in the control animals and in those receiving sulfathiazole; subsequent recovery was rapid.

The drugs as the free acids were administered by stomach tube to monkeys in a daily dose of 0.4 g per kilo body weight.† It was estimated that this dose represents about 3 times the maximum human therapeutic dose of sulfapyridine according to present recommendations. Sulfathiazole and sulfapyridine were fed to 3 monkeys each for 14 days, and to 4 each for 21 days. With one exception, the loss of weight was negligible in the monkeys receiving sulfathiazole, whereas it was definite and often pronounced in those given sulfapyridine. One monkey receiving sulfathiazole died on the eighteenth day apparently as a result of uremia due to the accumulation of acetylsulfathiazole in the collecting tubules; in addition, there was distension of the ureter on the left side because of an accretion of the conjugated drug in the ureter. No renal damage could be detected grossly or microscopically in the other 6 monkeys receiving sulfathiazole. Six of 7 monkeys receiving sulfapyridine began to exhibit hematuria and albuminuria after a few days and at necropsy were found to be suffering from unilateral or bilateral hydroureter and hydronephrosis, confirming the reports of others.[6] The chronic toxic effects of sulfathiazole in rats and monkeys are much less pro-

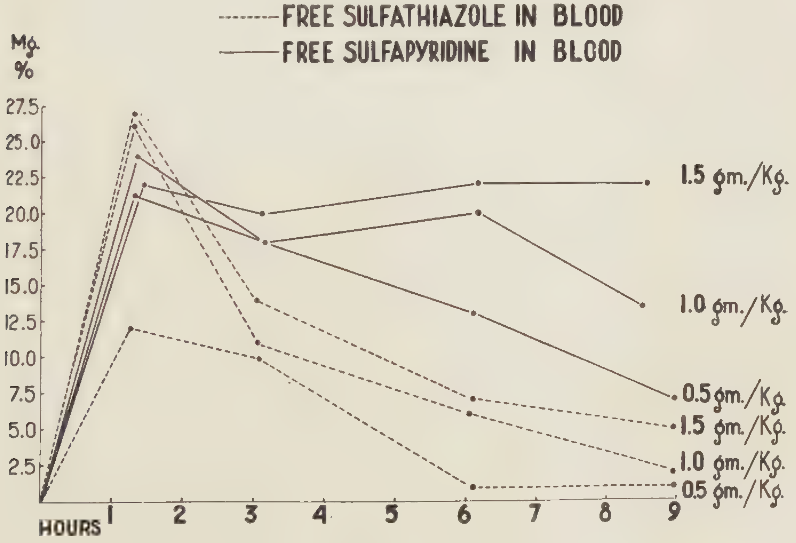

FIG. 2.

The level of sulfathiazole or sulfapyridine in the blood of mice after the oral administration of the free acids. See text.

Ordinate: concentration of drug in blood. Abscissa: time in hours.

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† These experiments were performed by Dr. Warren C. Corwin.

[6] Antopol, W., and Robinson, H., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 428, and others.

nounced probably because the drug as shown by our metabolic studies is more rapidly metabolized and undergoes much less conjugation than sulfapyridine.

Metabolic studies have been made in mice, rats, and monkeys. In Fig. 2, the levels of free sulfapyridine and sulfathiazole in the blood of mice are plotted against time in hours.[‡] The doses of the free acids as suspensions in gum acacia were administered by stomach tube. Nineteen of the points represent the average values of 5 animals each; the remaining 5 points are the average values of 4 animals each. It is evident that sulfathiazole disappears from the blood more rapidly than sulfapyridine; however, the data do not indicate whether this difference is due to more rapid destruction, more rapid tissue fixation, more rapid excretion, or to a combination of these factors. In a second experiment in mice, the sodium salts in a dose of 1 g per kilo body-weight were injected subcutaneously. At the end of 1, 3, and 6 hours the blood levels were: sulfapyridine, 74, 69, and 47 mg %; sulfathiazole, 90, 79, and 6 mg %. Fifty percent of the mice receiving sulfapyridine were dead at the end of 3 hours, whereas none of the mice receiving sulfathiazole had died spontaneously at the end of 6 hours. Such observations indicate that early deaths cannot be correlated with the levels of the drugs in the blood.

The urinary excretion of the drugs in groups of rats is summarized in experiment 1 of Table III. The total excretion of sulfathiazole was slightly greater in the 24-hour period. However, at the end of 4 and 8 hours the excretion of sulfathiazole had taken place at more than double the rate of excretion of sulfapyridine. During the succeeding 16 hours, sulfapyridine was excreted more and more rapidly. It is of great interest that, in comparison with sulfathiazole, about twice as much sulfapyridine was excreted in the form of the acetylated derivative.[||] These findings were confirmed and extended in monkeys (experiments 2-4, Table III) in which an even greater difference in the nature of the excretion-products was detected. The monkey excreted about 3 times as much acetylated sulfapyridine as acetylated sulfathiazole. Total excretion of sulfathiazole was always higher. Particularly in the first 24 hours the rate of excretion of sulfathiazole was approximately twice to 4 times that of sulfa-

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[‡] Only insignificant amounts of the drugs in the blood appeared to be conjugated. All determinations were made by the method of Bratton and Marshall, *J. Biol. Chem.*, 1939, **128**, 537.

[||] It is assumed that the conjugated forms excreted by rats are acetyl derivatives. Acetyl sulfathiazole was isolated from the urine of a monkey receiving sulfathiazole by stomach tube.

TABLE III.
Excretion of Sulfapyridine or Sulfathiazole in Urine of Rats and Monkeys.

Experiment	Drug	Dose, g/kg	Route	Period of observation, hr	% of dose recovered	Distribution of drug in urine	
						Free, %	Conjugated, %
1*	Na Sulfapyridine	0.75	Subcut.	24	25.9	55.8	44.2
	,, ,,		,,		24.2	58.2	41.8
	Na Sulfathiazole		,,		33.0	77.9	22.1
	,, ,,		,,		29.6	81.6	18.4
2	Sulfapyridine	1.00	,,	72	10.2	36.5	63.5
	Sulfathiazole		,,		23.3	86.2	13.8
3	Na Sulfapyridine	0.500	,,	96	55.6	39.6	60.4
	Na Sulfathiazole	0.513	,,		67.6	78.6	21.4
4	Sulfapyridine	1.000	Stomach tube	95	19.4	30.2	69.8
	Sulfathiazole	1.025	,, ,,		28.0	76.2	23.8

* There were 4 adult rats in each group in this experiment. All the remaining experiments were performed with single monkeys (*Macaca mulatta*).

TABLE IV.
Distribution of Sulfapyridine or Sulfathiazole in Blood of Monkeys.*

Experiment†	Drug	Interval after treatment, hr	Drug in blood		
			Total, mg %	Free, %	Conjugated, %
3 Subcutaneous injection	Na Sulfapyridine	1.1	5.1	78	22
	” ”	3.2	14.1	65	35
	” ”	6.1	17.0	63	37
	” ”	24.6	21.0	14	86
	” ”	48.0	5.7	19	81
	Na Sulfathiazole	1.1	11.7	88	12
	” ”	3.1	18.5	83	17
	” ”	6.1	15.3	84	16
4 By stomach tube	Sulfapyridine	2.9	4.0	50	50
	”	6.6	10.0	38	62
	”	23.3	11.3	8	92
	”	30.8	5.7	11	89
	Sulfathiazole	2.6	3.8	79	21
	”	6.3	4.8	75	25
	”	23.0	1.8	72	28

* No blood-levels below 1 mg % are included. In both experiments observations were concluded after 95-96 hours.

† See Table III for additional data on treatment.

pyridine. In experiment 3, for example, 96% of the total excretion of sulfathiazole occurred within 24 hours after injection whereas 61% of the total sulfapyridine excreted was found in the urine during the succeeding 24-hour periods.

In the monkey the proportion of free sulfathiazole in the blood is also much higher than that of free sulfapyridine (Table IV). This is especially noteworthy in experiment 4 after administration of the free drugs by stomach tube. Although, contrary to our other experiments, the levels of total sulfapyridine are usually the higher, the levels of free drugs are essentially alike, indicating that sulfapyridine would here have no chemotherapeutic advantage since the acetylated form is valueless.

Summary. If the sodium salts of the two drugs be used, the toxicity of sulfathiazole appears to be about 65% of the toxicity of sulfapyridine for LD50. Repeated administration of the drugs in the food of mice indicates that sulfathiazole is more toxic than sulfapyridine at a high dose level but that there is no difference at a dose level which is effective therapeutically. In monkeys and growing rats, receiving either drug for 14-57 days, sulfapyridine is clearly the more toxic. The principal pathological change in all 3 species appears to be renal damage.

Metabolic studies indicate that sulfathiazole is more rapidly metabolized and undergoes much less conjugation than sulfapyridine.

10922

Therapeutic Effect of Sulfathiazole and Sulfapyridine.

C. M. McKee, Geoffrey Rake, R. O. Greep and H. B. van Dyke.
From the Squibb Institute for Medical Research, New Brunswick, N. J.

The pharmacology of the new thiazole analogue of sulfapyridine prepared by Lott and Bergeim[1] has been described in a preceding paper.[2] The therapeutic effect of this drug has been tested in a large number of mice against several different infections. In all cases the effect has been measured against that produced by the same amounts of sulfapyridine. The results are discussed below.

The drugs have been administered by several different methods. Thus the free acid has been injected by stomach tube as a suspension both in water and in gum acacia and also subcutaneously as an aqueous suspension. A solution of the sodium salt in water has been given by stomach tube. The results obtained when the drugs were given by any of these methods indicated that the new compound, sulfathiazole, was very promising (Table I). Several *in vitro* tests also gave results promising for sulfathiazole. However, the physical properties of sulfathiazole are such that its administration as a suspension proved to be technically difficult. The insoluble powder settled out more rapidly than did the sulfapyridine, rendering it difficult to give the full doses indicated or to measure the actual dose given. Moreover, in certain experiments the sodium salt was found to be precipitated in the stomach in the form of a curd much of which remained for several days so that the mice did not absorb the anticipated amount of drug. The technical difficulties outlined above might account for some of the less favorable results obtained with sulfathiazole as compared with sulfapyridine (Table I). In addition, however, was the fact that sulfathiazole disappears from the circulating blood after single administrations much more rapidly than does sulfapyridine.[2] In all the above experiments the drugs were administered twice a day at 9 a.m. and 5 p.m., leaving an interval of 16 hours when no drug was given and the blood level of sulfathiazole probably dropped to low levels. It was thought that better results would be obtained were more repeated administration assured and it was, therefore, decided to adopt the method of mixing the drug with

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[1] Fosbinder, R. J., and Walter, L. A., *J. Am. Chem. Soc.*, 1939, **61**, 2032; Lott, W. A., and Bergeim, Frank H., *J. Am. Chem. Soc.*, in press.

[2] van Dyke, H. B., Greep, R. O., Rake, Geoffrey, and McKee, C. M., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 410.

TABLE I.

				Number of mice treated with								
				Sulfathiazole			Sulfapyridine			Untreated		
	Therapy				Died			Died			Died	
Infecting organism	Route of administration	Amt per day mg/k	Days treatment	Lived, No.	No.	Avg life in days	Lived, No.	No.	Avg life in days	Lived, No.	No.	Avg life in days
Pneumococcus Type 1 P	*subcu.	1000	5	3	27	4.6	1	29	3.9	0	30	<1
Type 2 P	"	"	"	6	24	6.5	18	12	9.4	0	30	<1
Type 3 S	"	"	"	10	17	5.8	21	9	10.9	0	30	1.7
Meningococcus Type 1	"	"	"	11	18	4.0	8	20	2.1	0	29	1.4
Pneumococcus Type 1 P	†oral	1250	10	6	19	8.5	13	14	8.6	0	9	1.4
Lymphogranuloma venereum	"	"	7	13	0		13	0		11	19	8.9

* Suspension of free acid in distilled water.

† Suspension of free acid in gum acacia.

the food as described by Bieter and coworkers[3] and Litchfield and coworkers.[4]

The drugs were added in 1% concentrations to Sherman mouse-diet No. 1. Mice were placed on the diet 2 days before they were infected so that they would become accustomed to the food. Our observations agree with those of other workers[3, 4] that mice eat such a diet freely throughout the 24 hours with a peak of consumption around midnight and lowest consumption around 6 a.m. The amount of drug consumed daily by this method is approximately equal for sulfathiazole and sulfapyridine and averages around 1.85 g per kilo. Following infection mice were maintained on the drug diet for 10 days and were then returned to normal diet for a further 10 days' observations. All results are given up to the termination of the 20 days.

Infections with the following organisms have been tested: Pneumococcus Types 1 (4 strains), 2 (3 strains), 3 (5 strains), 5, 6, 7, 8, 14, and 26, meningococcus, and *Streptococcus hemolyticus.* All infection was by the intraäbdominal route. The dose varied, but for the pneumococcal strains was usually one cc of a $1:10^{-7}$ dilution of a 5-hour blood-broth culture. All strains used have been maintained in a state of constant virulence by frequent mouse-passage.

Table II shows representative results. It will be noted that there is little difference between the two drugs. In the case of some strains, *i. e.,* pneumococcus Type 2-807 and pneumococcus Type 8, little protection occurred with either drug; in others, *i. e.,* pneumococcus 7 and the *Streptococcus hemolyticus*—C203, perfect protection was obtained with both drugs.

In the few experiments in which 0.5% of the drugs have been used in the diet the results with sulfathiazole have been slightly but consistently less favorable than those with sulfapyridine, a result to be expected in view of what occurs to the blood-levels of the two drugs in mice on a 1% diet. Single experiments on mice fed the drug in the diet, and the blood-curves following injection[2] indicate that the sulfathiazole blood-level is more dependent on the intake of drug in the food. During periods of low food-consumption the level of sulfathiazole will be lower than that of sulfapyridine; at other times the level of the two drugs is probably similar. Only representative experiments have been given in any detail in the tables and they show only a part of the work which has been carried out.

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[3] Bieter, R. N., Larson, W. P., Cranston, E. M., and Levine, Milton, *J. Pharm. and Exp. Ther. Proc.*, 1939, **66**, 3.

[4] Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *J. Pharm. and Exp. Ther. Proc.*, 1939, **66**, 23.

TABLE II.

Infecting organism*	Number of mice treated with								
	Sulfathiazole diet†			Sulfapyridine diet†			Normal diet		
		Died			Died			Died	
	Lived No.	No.	Avg life in days	Lived, No.	No.	Avg life in days	Lived, No.	No.	Avg life in days
Pneumococcus Type 1 P	15	31	3.3	14	31	3.9	0	38	.82
,, 748	15	5	6.	15	5	5.1	0	20	1.66
,, 2 P	16	8	11.8	21	3	6.3	0	23	.77
,, 807	0	20	2.2	0	20	2.	0	20	< .75
,, 3 S	21	1	15.	21	1	9.	1	21	1.8
,, 750	1	19	7.	3	17	6.2	0	20	.97
,, 5 752	17	3	5.2	16	4	3.	0	20	.9
,, 6	8	12	11.2	13	7	16.7	0	20	1.37
,, 7 754	20	0		20	0		0	20	1.49
,, 8 755	1	19	3.7	0	20	4.2	1	13	1.4
,, 14 848	10	0		10	0		1	7	1.7
,, 26	14	6	13.3	11	9	13.4	1	19	2.79
Meningococcus Type II	16	2	1.5	12	6	2.3	1	9	1.0
Streptococcus hemolyticus	14	0		13	0		0	14	.9

* Dose varied from 10^{-5} to 10^{-7} of a 5-hour culture depending on virulence.

† One percent drug in food.

Thus, 650 mice have been used in tests of the efficacy of the drugs when administered by methods other than that of addition to the diet. Approximately 1200 mice have been used in experiments with a 1% diet and 200 on a 0.5% diet.

As has been shown in Table I both sulfathiazole and sulfapyridine have an appreciable therapeutic effect against the agent of *lymphogranuloma venereum*. In tests with this agent 85 mice have been used. These results are in agreement with those obtained by others with sulfanilamide. Neither sulfathiazole nor sulfapyridine, however, has shown any activity against swine-influenza virus or *herpes simplex*. In these tests 300 mice have been used. Further virus-studies are in progress.

Conclusions. It has been shown that when the compounds which have been studied are administered as 1% of the diet, the therapeutic effect of sulfathiazole is equal to that of sulfapyridine. In view of the comparative toxicity and metabolism of these two compounds[2] it is possible that sulfathiazole may be a more desirable therapeutic agent than sulfapyridine.

10923

Chemotherapeutic Evaluation of Sulfanilamide Derivatives of Heterocyclic Amines.

FRANK B. COOPER, PAUL GROSS AND MARION LEWIS.
From the Western Pennsylvania Hospital Institute of Pathology, Pittsburgh, Pa.

The therapeutic success with which sulfanilamide has been used in a number of unrelated bacterial and several viral diseases has stimulated the search for more effective and less toxic drugs. The trend of these investigations has generally been to modify the sulfanilamide nucleus by substitution in the amine or amide group, replacement of the amide group or degradation of the sulfone group. These substitutions have generally been of the alkyl, aryl, or substituted-alkyl or substituted-aryl type. The few compounds formed by the substitution of heterocyclic radicals for amino or amide hydrogens were of no especial interest, prior to the advent of sulfapyridine, because of their relatively feeble therapeutic activity.

Of these, the heterocyclic azo compounds *p*-sulfonamidophenylazodihydrocupreine,[1, 2] *p*-sulfonamidophenylazoapoquinine,[2] *p*-sulfonam-

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[1] Heidelberger, M., and Jacobs, W. A., *J. Am. Chem. Soc.*, 1919, **41**, 2145.

[2] Buttle, G. A. H., Gray, W. H., and Stephenson, D., *Lancet*, 1936, **1**, 1286.

idophenylazoisoapoquinine,[2] and *p*-sulfonamidophenylazoisoquinidine[2]and also tropinone bis-sulfonamideophenylhydrazone[2] possessed very litle therapeutic activity.

The amino substituted compounds *p*-furfurylideneaminophenylsulfonamide[3] and 2-pyrrolidone-5-carboxy-4′-aminophenylsulfonamide[3] were claimed to be respectively less active and more active than sulfanilamide in streptococcic infections.

The two nitrogen-linked sulfones, l-sulfanilylpiperidine[3] and l-sulfanilylpiperazine* were inactive, whereas 2-pyrrolidone-5-carboxy-4′-amino-phenylsulfinic acid[4] was reported to be as active as sulfanilamide.

The amide substituted compounds, *p*-aminophenylpiperidylsulfonamide[5] and 2-sulfanilamidopyridine (sulfapyridine)[6] were claimed to be respectively one-half as active and as good as or better than sulfanilamide against streptococcic infections. The latter compound, in adequate dosage, was claimed by Whitby[6] to save all mice infected intraperitoneally with 10,000 fatal doses of Types I, VII, and VIII pneumococci.

Subsequent investigations have shown sulfapyridine to be fractionally superior to sulfanilamide against mouse infections of approximately 10 to 1000 fatal doses of pneumococci[7–11] and of almost equal efficacy against experimental pneumococcus pneumonia[11] and meningitis[7, 11] of rats.

Even though the demonstrated superiority of sulfapyridine over sulfanilamide when used in equal doses in experimental infections, was considerably less than that originally claimed for it, this slight improvement suggested the possibility that more effective therapeutic agents might result from other heterocyclic substitutions of sulfanilamide. Accordingly, the following 5 compounds,† which contain component nuclei characteristic of other well known medicinals,

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[3] Gray, W. H., Buttle, G. A. H., and Stephenson, D., *Biochem. J.*, 1937, **31**, 724.

* Synthesized and donated by the Monsanto Chemical Company, St. Louis, Mo.

[4] Buttle, G. A. H., Dewing, T., Foster, G. E., Gray, W. H., Smith, S., and Stephenson, D., *Biochem. J.*, 1938, **32**, 1101.

[5] Poulain, P., *These de Paris*, 1936.

[6] Whitby, L. E. H., *Lancet*, 1938, **1**, 1210.

[7] Cooper, F. B., Gross, P., and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 37.

[8] Hilles, C., and Schmidt, L. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 73.

[9] Long, P. H., Bliss, E. A., and Feinstone, W. H., *Pennsylvania M. J.*, 1939, **42**, 483.

[10] Schmidt, L. H., and Hilles, C., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 611.

[11] Cooper, F. B., Gross, P., and Lewis, M., *J. Clin. Invest.*, 1939, **18**, 423.

† Synthesized and donated by the Maltbie Chemical Company, Newark, N. J.

TABLE I.
Streptococcic Infection of Mice (1,000-10,000 Fatal Doses).

	Treatment	No. of Mice	No. of deaths daily during 3 weeks									No. of Survivors	% of Survivors
			1	2	3	4	5	6	7	8	9-21		
None		50	32	7	3	1	1	1	2	2	1	0	0
I	Sulfanilamide	50		4		1		1	1		5	38	76
II	Sulfapyridine	30			1		1					28	93
III	2,6-Diamino-3-p-sulfonamidophenylazopyridine	10	3			2		1			2	2	20
IV	2-N4-acetylsulfanilamido-6-aminopyridine	10				2			2			6	60
V	2-Sulfanilamido-6-aminopyridine	40	4	1	2			1	1		3	28	70
VI	2-Sulfanilamidothiazole	30	1	1	1		1	1			2	23	77
VII	2-Sulfanilamido-4-methylthiazole	30	1			1			1		2	25	83

Infection: 0.5 cc of a 10–4 broth dilution of an 18-hour broth culture (C 203) intraperitoneally (1,000-10,000 fatal doses).

Treatment: 10 mg of drug suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 3 successive days (total 40 mg).

TABLE II.
Streptococcic Infection of Mice (100,000-1,000,000 Fatal Doses).

	Treatment	No. of Mice	No. of deaths daily during 3 weeks									No. of Survivors	% of Survivors
			1	2	3	4	5	6	7	8	9-21		
None		25	25									0	0
I	Sulfanilamide	25	20	2							1	2	8
II	Sulfapyridine	25	13	2	1							9	36
V	2-Sulfanilamido-6-aminopyridine	25	19							1	1	4	16
VI	2-Sulfanilamidothiazole	25	18						2			5	20
VII	2-Sulfanilamido-4-methylthiazole	25	17	1						1	1	5	20

Infection: 0.5 cc of a 10–2 broth dilution of an 18-hour broth culture (C 203) intraperitoneally (100,000 to 1,000,000 fatal doses).

Treatment: 10 mg of drug suspended in 0.2 cc of gum acacia orally 3 hours after infection, then once daily for 3 successive days (total 40 mg).

TABLE III.
Type II Pneumococcic Infection of Mice (100 Fatal Doses).

Treatment		No. of Mice	No. of deaths daily during 3 weeks									No. of Survivors	% of Survivors
			1	2	3	4	5	6	7	8	9-21		
None		30	2	10	13	4	1					0	0
I	Sulfanilamide	30		1		1	3	3	7	1	2	12	40
II	Sulfapyridine	30			1		1		4	2	4	18	60
III	2,6-Diamino-3-p-sulfonamidophenylazopyridine	10	6	1	3							0	0
IV	2-N⁴-acetylsulfanilamido-6-aminopyridine	10	1	3	4	2						0	0
V	2-Sulfanilamido-6-aminopyridine	30	4	4		1				3	3	15	50
VI	2-Sulfanilamidothiazole	30	1	1			5		1	6	3	13	43
VII	2-Sulfanilamido-4-methylthiazole	30			1			2	4	3	6	14	47

Infection: 0.5 cc of a 10^{-6} broth dilution of an 18-hour broth culture of Type II (Binda) subcutaneously (100 fatal doses).

Treatment: 20 mg of drug suspended in 0.2 cc of 15% gum acacia orally 3 hours after infection, then once daily for 5 successive days (total 120 mg).

were synthesized[12] and their antistreptococcic and antipneumococcic activities compared to those of sulfanilamide and sulfapyridine: 2,6-diamino-3-p-sulfonamidophenylazopyridine; 2-N^4-acetylsulfanilamido-6-aminopyridine; 2-sulfanilamido-6-aminopyridine; 2-sulfanilamidothiazole; 2-sulfanilamido-4-methylthiazole.

The therapeutic activities of these compounds were determined by inoculating mice with a number of infecting doses just within or slightly beyond the effective range of sulfanilamide and sulfapyridine therapy and maintaining the treatment somewhat below the optimum level. In this manner, differences in drug efficacy are more readily seen than when all animals in two or more treated groups survive.[13] Thus, the values obtained do not indicate the maximum number of survivors which would have been obtained under optimum treatment but rather the relative values of the various drugs over an adequate period of observation.

Tables I, II, and III show sulfapyridine to be somewhat superior to compounds V, VI,‡ and VII,‡ which possess approximately the same degree of therapeutic activity as sulfanilamide, against streptococcic and Type II pneumococcic infection in mice.

Main, Shinn and Mellon[14] have reported that bacteriostasis and the accompanying increased accumulation of hydrogen peroxide per unit of growth were similar in cultures of Type I pneumococcus which contained comparable amounts of sulfanilamide, sulfapyridine, VI or VII.

10924 P

Associative Hysteresis in Larval Amblystoma.

A. R. Moore and J. C. Welch.

From the Department of Psychology, University of Oregon, Eugene.

The object of the study was to determine whether larval amblystoma are able to establish associations with non-essential signals, *i. e.*, are capable of true learning. This necessarily rules out such effects of training as facilitation of primary reflexes. It was found that the larvae of *Amblystoma paroticum* could form associations between food taking and the movement of an object in the visual field. In response to movement above them, as, passing the hand back and forth, they would rise from the floor to the top of the aquarium. In the dark room, they did not form associations with the turning on of a constant light, but did learn to respond to intermittent flashes, 52 to the minute. They also learned a simple T-maze of which one end was closed, the other open, allowing the animal to slip down into a dish of water. This constituted the reward. Five animals were given in all 537 trials in 9 experimental series. Each animal in at least one series learned to take the "correct" turn. The results of each series were subjected to statistical analysis, which showed that of the 9 experiments, 2 fall within the probability of the results being due to chance alone, the remaining 7 unquestionably show the effects of learning. The third type of reaction studied was the snap reflex. It was attempted to bring about inhibition of this reflex. For this purpose an earthworm was put into a large glass tube and, in a test, the preparation was held in front of the salamander for 5 minutes and the observer counted the number of strikes made during the period. Two tests a day were given. Two larval axolotls were used. On the first day one of them struck 14 times and the other 23. By the fourth day extinction of the reflex was almost complete, one animal striking once, the other not at all. After this inhibition had become established, holes were made in the glass tube to allow sapid substances from the worm to diffuse out. The salamanders now attacked more vigorously and repeatedly than they had done in the initial test, *i. e.,* to visual stimuli alone. It now required 12 days for extinction of the reflex. This shows the olfactory to be more powerful than the visual stimulus in exciting the snap reflex. Complete inhibition of the snap reflex to both types of stimulus can be brought about. It is concluded that three types of simple associations can be established in larval amblystoma.

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[12] Fosbinder, R. J., and Walter, L. A., *J. Am. Chem. Soc.*, 1939, **61**, 2032.

[13] Cooper, F. B., Gross, P., and Lewis, M., *J. Chemotherapy*, 1938, **15**, 31.

‡ These drugs, like sulfapyridine, produce renal concrements in rats. A detailed study of these, and other toxicological data will be published shortly.

[14] Main, E. R., Shinn, L. E., and Mellon, R. R., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 115.

10925

The Micro Flocculation Test of Eagle in Syphilitic White Mice.

ELSIE MAE FITZGERALD, MILDRED SHEPHERD AND JAROLD E. KEMP.
From the Research Division of the Public Health Institute, Chicago, Ill.

A review of the literature shows that reports of the results of the serologic testing of syphilitic white mice are in close agreement. In a study of 93 such mice, Karrenberg[1] and Kertopati[2] each observed that only 4.3% gave a positive micro Meinicke reaction. Three of Kertopati's positive mice were known to have negative tests prior to inoculation. Using the same test, Schumacher[3] found that only 7% of over 300 syphilitic mice were consistently positive over a period of from 3 to 7 months after inoculation, and that the Wassermann reaction was positive in about the same percentage. Zih[4] and Koch,[5] however, reported completely negative results with the micro Meinicke test in a total of 64 and 98 syphilitic mice, respectively. The duration of the infection at the time Zih's mice were tested varied from two weeks to 20 months.

In this experiment, 57 adult white mice were inoculated intraperitoneally with from 0.1 to 0.2 cc of an emulsion of a rabbit testicular syphiloma containing approximately 3 to 20 treponemes per high power dark field. The Nichols strain of *T. pallidum* was used. These mice were tested prior to inoculation and found to be entirely negative. The tests were repeated 30, 60, and 120 days after inoculation. Only 38 animals survived for 120 days. Since in our experiences intraperitoneal inoculations with the Nichols strain of *T. pallidum* are successful in 90% of mice as judged by the infectiousness of their brains for normal rabbits, no attempt was made to prove that these mice had been successfully inoculated. The micro Eagle test[6] was used because of the small amount of blood required for the test and because of its reliability in our hands.

Under ether anesthesia about 0.5 cc of blood was obtained from each mouse by excision of the tip of the tail. The blood was collected in small glass tubes approximately 5 mm in diameter. After coagulation it was centrifuged at 2,000 r.p.m. for 20 minutes, following which 0.1 cc of the serum was removed with a capillary pipette and inactivated in a water bath at 56°C for 30 minutes. 0.05 cc of antigen[7] was then added and the mixture shaken for 5 minutes. It was then incubated for 30 minutes, and the results, both macro- and microscopic, read immediately thereafter.

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[1] Karrenberg, C. L., *Arch. f. Dermat. u. Syph.*, 1932, **165**, 605.

[2] Kertopati, S., *Dermat. Z.*, 1931, **62**, 174.

[3] Schumacher, C., *Dermat. Z.*, 1932, **64**, 289.

[4] Zih, A., *Med. Klin.*, 1929, **25**, 431.

[5] Koch, F., *Dermat. Z.*, 1933, **65**, 24.

[6] Eagle, H., and Brand, A. F., *Am. J. Syph., Gonorr. and Ven. Dis.*, 1938, **22**, 22.

TABLE I.
Results of the Micro Eagle Test in Syphilitic White Mice.

Duration of infection days	No. of mice	Results	
		Positive	Negative
30	28	0 0%	28 100%
60	47	2 4.3%	45 95.7%
120	38	1 2.6%	37 97.4%

As shown in Table I, the test was entirely negative in 28 mice 30 days after inoculation. Two or 4.3% of 47 mice were positive 60 days after inoculation and 1 or 2.6% of 38 mice was positive 120 days after inoculation. Of the 2 mice that were positive 60 days after inoculation one became negative when tested 120 days after inoculation; the other was still positive at the end of 120 days.

Summary and Conclusions. In a study of the micro Eagle test on the sera of 57 syphilitic white mice, we found that: (1) of 28 mice infected 30 days, none was positive; (2) of 47 mice infected 60 days 2 or 4.3% were positive; and (3) of 38 mice infected 120 days 1 or 2.6% was positive. These findings confirm those of other authors; namely, that a positive serologic test for syphilis infrequently occurs in the syphilitic mouse and is, therefore, of no value in studying the course of experimental syphilis in this animal.

10926

A New Method for Quantitative Measurement of Pain Sensation.

DAVID I. MACHT AND MOSES B. MACHT.

From the Pharmacological Research Laboratory, Hynson, Westcott & Dunning, Inc., and the Department of Psychology, Johns Hopkins University, Baltimore, Md.

A suitable method for quantitative measurement of analgesia (as distinguished from local anesthesia) produced by various physical and chemical agents is a crying need of modern experimental pharmacology, physiology and psychology. The method of Macht, Herman and Levy[1, 2] for determining pain threshold in normal human subjects is probably the most suitable for such drugs as the opium alkaloids, which have a wide clinical use and fully established margin of safety. Animals being indispensable for quantitative study of new and little known compounds, however, the authors have developed a "rat test" employed in the following manner.

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[7] Eagle, H., *J. Lab. and Clin. Med.*, 1936, **22**, 300.

The scrotum of a large tame male rat is a very suitable test object for the quantitative measurements of pain sensation. Richly supplied with sensory nerves, it readily responds to pain stimuli produced by a faradic current. When fine platinum electrodes connected with a standardized induction coil are applied to any given spot on the scrotum of an adult white rat and the current is increased by gradually approximating the secondary to the primary coil, a point is reached when the stimulation is sufficiently powerful to produce pain. This point, indicated by the animal's characteristic squeal, is the pain threshold for that given area, and numerous experiments performed by the authors have proven that it is surprisingly constant. This method, therefore, admits of careful study of the effect produced upon the pain threshold by various pharmacodynamic agents.

In this work the writers employed a standard Harvard inductorium operated on a single dry cell of 1.6 volts, which was carefully calibrated in the physical laboratory of a well-known university, so that the intensity of the stimulations for any given position of the secondary coil in relation to the primary could be expressed in physical units, *viz.*, volts. The inductorium was calibrated with the aid of an R.C.A. cathode ray oscillograph, and a curve was plotted to express the voltage corresponding to any given position of the secondary coil. The fine electrodes are applied to the scrotum of a large tame rat, held gently but firmly by an assistant, at a point marked for identification. The pain threshold is then established by slowly increasing the faradic current until the animal's characteristic squeal of pain is elicited. When the threshold of pain has been accurately established by repeated trials, the authors administer a drug by subcutaneous or intramuscular injection or, if the compound is insoluble, by stomach tube. The effect of the drug on the pain threshold is then measured at various intervals after its administration.

The series of drugs tested on rats in this way included various opium alkaloids, coal tar derivatives, barbiturates, bromides, etc., on

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[1] Macht, D. I., Herman, N. B., and Levy, C. S., *Proc. Nat. Acad. Sc.*, 1915, **1**, 582.

[2] Macht, D. I., Herman, N. B., and Levy, C. S., *J. Pharm. and Exp. Therap.*, 1916, **8**, 1.

TABLE I.
Comparison of Effects of Morphine and Other Analgesics.

Rat No.	Drug administered	Dose given mg	Pain Threshold							
			Normal		15-30 min after injection		1-2 hr after injection		4 hr after injection	
			cm	volts	cm	volts	cm	volts	cm	volts
1	Morphine Sulfate	1	8.0	185	7.6	250	7.8	218	—	—
2	,, ,,	1.5	7.6	250	6.6	530	7.1	350	7.7	232
3	,, ,,	3	7.1	350	5.4	1080	5.8	953	7.7	232
4	Codeine HCl	2	9.5	125	8.2	176	9.5	125	—	—
5	,, ,,	3	8.0	185	7.6	250	8.0	185	—	—
6	Pantopon	2	8.0	185	6.5	575	7.3	300	7.6	250
7	Dilaudid	0.25	9.0	100	7.0	368	7.0	368	—	—
							1 hr after injection		2 hr after injection	
8	Acetanilid	4	7.8	218	7.8	218	7.0	368	7.5	265
9	Antipyrin	10	7.6	250	—	—	7.2	325	7.3	300
10	Caffeine	4	8.1	180	8.1	180	8.4	170	—	—
11	Cocaine HCl	2	9.5	125	9.8	110	9.8	110	—	—
12	Phenolbarbital Na	5	7.4	280	—	—	7.8	218	7.7	232
13	Salicylate Na	10	7.2	325	6.8	445	6.8	445	—	—
14	Strychnine Nitr.	0.1	7.3	300	8.0	185	8.2	176	—	—
					45 min after administration		2 hr after administration		3 hr after administration	
15	Acetphenetidin (by stomach tube)	30	8.8	155	7.5	265	7.6	250	—	—

TABLE II.
Comparison of Analgesic Effects of Cobra Venom and Morphine.

Rat No.	Drug administered	Dose injected	Pain Threshold							
			Normal		45 min after injection		2 hr after injection		4·5 hr after injection	
			cm	volts	cm	volts	cm	volts	cm	volts
1	Morphine Sulfate	1 mg	6.0	809	6.1	770	7.0	368	6.0	809
2	” ”	1 ”	6.4	630	6.6	530	7.4	280	6.4	630
3	” ”	1 ”	6.5	575	6.0	809	6.0	809	6.5	575
4	” ”	1 ”	8.0	185	7.2	325	7.2	325	8.0	185
5	Cobra Venom	0.5 m.u.	7.0	368	6.6	530	6.7	480	6.5	575
6	” ”	0.5 ”	8.3	173	7.5	265	7.2	325	7.2	325
7	” ”	0.5 ”	8.3	173	6.8	445	6.3	665	6.2	715
8	” ”	0.5 ”	7.2	325	6.5	575	6.1	770	6.0	809
9	Morphine Sulfate	3 mg	7.9	205	4.9	1260	8.0	185	7.9	205
10	” ”	3 ”	7.1	350	5.4	1080	7.9	205	7.2	325
11	” ”	3 ”	8.5	166	6.5	575	8.4	170	8.4	170
12	” ”	3 ”	8.0	185	6.8	445	8.3	173	8.0	185
13	Cobra Venom	1 m.u.	8.0	185	7.0	368	4.0	1932	5.0	1223
14	” ”	1.5 ”	8.2	176	8.4	170	7.2	325	6.4	630
15	” ”	1.5 ”	7.1	350	6.8	575	6.8	575	5.8	953
16	” ”	2 ”	8.0	185	6.0	809	5.4	1080	6.4	630

the one hand, and, on the other, drugs used as controls, such as strychnine and caffeine. It was found that some of the drugs *raised* the pain threshold, that is, produced analgesia. Other drugs *lowered* it, thus indicating hyperalgesia or hypersensitivity to pain. Table I shows the results obtained with a number of such drugs. It will be seen that morphine, codeine, pantopon and dilaudid, antipyrin and acetphenetidin produce varying degrees of analgesia. Sodium phenobarbital, 5 mg, did not relieve pain while 10 mg of sodium salicylate effected some analgesia. Strychnine and caffeine lowered the threshold of pain. It was not surprising to find that the local anesthetic cocaine, which is a delirifacient when injected parenterally, induced hyperalgesia. It will be noted that the degree of analgesia produced by morphine varied with individual rats.

Of special interest were the results obtained in a comparative study of morphine and cobra venom, an analgesic now used considerably in place of the narcotics.[3, 4] Table II shows that morphine in sufficiently large doses produces an analgesia that sets in promptly after administration but wears off in a short while. The analgesia induced in rats by cobra venom, on the contrary, supervenes but slowly and lasts much longer than that of morphine. These findings regarding the effect of morphine and cobra venom, respectively, on the pain threshold of rats agree with those derived by Macht and Bryan from studies on the threshold of pain of human subjects and also of guinea pigs.[5] The results obtained by this method in regard to the comparative analgesic properties of various well-known drugs completely concur with clinical data concerning them. This new method for quantitative measurement of pain threshold and analgesia was furthermore found to be useful in the study of new and unknown compounds believed to possess pain-relieving properties such as, for instance, the various fractions obtained in connection with a biochemical study of snake venoms. A more detailed discussion of the technic employed and of the effects of numerous drugs as studied by this method will appear in the *Journal of the American Pharmaceutical Association.*

Summary. A new method for quantitative determination of pain threshold in rats is described. The results obtained with various analgesic drugs, including morphine and cobra venom, are cited as illustrations of its practicability.

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[3] Macht, D. I., *Ann. Int. Med.*, 1938, **11**, 1824.

[4] Macht, D. I., *M. Press*, 1939, **201**, 254.

[5] Macht, D. I., and Bryan, H. F., *Compt. rend. Soc. de biol.*, 1935, **119**, 306.

10927 P

Effect of Repeated Injections of Cobra Venom and Morphine on Behavior of Rats in a Maze.

Moses B. Macht. (Introduced by David I. Macht.)

From the Department of Psychology, Johns Hopkins University, and the Pharmacological Research Laboratory, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

Some years ago Macht and Mora found that injections of morphine induced a primary excitation followed by secondary depression in albino rats trained to run in a circular maze.[1] More recently, Macht and Bryan, studying the effects of cobra venom, an analgesic often substituted for morphine, on behavior of rats in Watson's maze, found that small doses of this drug[2] also excited the animals and that after such primary stimulation, larger doses of the venom produced depression. In the present study the author investigated the respective effects on behavior of rats of *repeated* doses of cobra venom and morphine, injected for long periods, in order to obtain more light on the problem of habituation and other questions connected with continual administration of pain-relieving drugs.

Studies were made on the behavior of 20 male albino rats averaging 225 g in weight. The rats were trained to run in Watson's circular maze until no learning effect was exhibited or until further training resulted in neither a lowering of the running time or reduction in number of errors made. After the optimum learning effect has thus been attained, the time required by each rat to run the maze and the number of errors it made both before and after injection of the drug studied were carefully determined. Before and after receiving the drug each rat was given 5 runs, the first 2 merely to preclude any diurnal learning processes. The time and number of errors made in the last 3 runs were noted and the average performance of each rat per minute was recorded. Daily injections of morphine were given half the series and of cobra venom to the remaining 10 rats for several weeks, at the end of which time the whole series rested for 2 weeks. When the rest period had expired, the procedure was reversed, and injections of morphine were given to the half series that had had cobra venom and of cobra venom to the 10 rats which had received morphine. In this way the comparative effect of both morphine and cobra venom was studied on the same animals. It was found that 0.5 mouse unit of cobra venom either had no effect at all or but slightly improved the usual performance of the rats. Larger doses, 2 mouse units or more, of cobra venom effected a primary stimulation succeeded by depression from which, however, the rats fully recovered on the following day. On the other hand, repeated daily injections of 0.5 mouse units or even of 1.0 mouse unit for periods of 2 weeks or more established no habituation as evidenced by the fact that there was no significant change in running performance. Neither was any cumulative effect observed after such a series of injections.

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[1] Macht, D. I., and Mora, C. F., *J. Pharm. and Exp. Therap.*, 1920, **16**, 219.

[2] Macht, D. I., *Proc. Nat. Acad. Sc.*, 1936, **22**, 61.

The results obtained with similar injections of morphine were quite different and very interesting. Three milligrams of morphine were found to be the smallest effective dose in the breed of rats studied. When such a dose of the narcotic was injected intraperitoneally, the rats were markedly depressed. When the same dose was administered on succeeding days, however, the rats showed less and less depression until finally no effect was produced by the narcotic or even a slight stimulation was noted. This tolerance varied somewhat with individual rats but in most cases was quickly achieved, a fact which may be explained by the recent finding that morphine is quickly oxidized by the brain tissue of the rat.[3] When the morphine dosage was increased to 5 mg, depression was first produced but as injections of this amount continued, the animals acquired a tolerance for this dose also and soon gave in their behavior no evidence of a narcotic effect. Table I is a complete protocol illustrating the findings made throughout the experiments with a rat selected at random from the series tested, and strikingly exhibiting the difference in the animal's behavior after repeated injections of morphine, on the one hand, and (a rest period intervening) after similar treatment with cobra venom, on the other. Similar results were obtained with all the rats in the series. These observations led to the conclusion that repeated injections of morphine induced tolerance or habituation in rats, as indicated by their behavior in a maze, whereas similar treatment with cobra venom had no such effect. These findings are in complete agreement with the clinical observations which numerous physicians have made on adults.[4, 5] Further data will appear in a more complete paper to be published in the *Journal of Experimental Psychology.*

Summary. Repeated injections of morphine induced in rats running in a circular maze a tolerance or habituation so that although

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[3] Macht, D. I., and Bryan, H. F., *Compt. rend. Soc. de Urol.*, 1936, **123**, 385.

[4] Macht, D. I., *Ann. Int. Med.*, 1938, **11**, 1824.

[5] Macht, D. I., *M. Press*, 1939, **201**, 254.

TABLE I.

Behavior of Rat B After Repeated Injections of Morphine and Cobra Venom, Respectively.

1939	Before administration								30 to 45 min. after administration							Wt in g
	Seconds			Avg	Errors			Dosage	Seconds			Avg	Errors			
4/30	8.1	7.8	6.0	7.3	0	0	0	—								210
5/1	8.1	6.5	6.3	6.9	1	0	0	—								
5/2	6.0	6.9	8.5	7.1	0	0	0	—								
5/3	9.0	6.4	6.0	7.1	0	0	0	—								
5/4	5.9	6.7	7.4	6.7	0	0	0	—								
5/5	7.0	6.0	6.0	6.3	0	0	0	—								212
								Morphine Sulphate								
5/7	8.0	7.0	7.0	6.7	0	0	0	3 mg	60.0	60.0	stalled	60.0+	2	5	—	
5/8	7.8	7.5	7.0	7.4	0	0	0	3 ”	16.2	27.1	45.1	32.8	2	2	2	
5/9	6.0	6.0	7.0	6.3	0	0	0	3 ”	9.2	8.8	9.0	9.0	1	0	0	
5/10	6.0	5.8	6.0	6.0	0	0	0	3 ”	6.1	5.8	6.0	6.0	0	0	0	
5/11	7.0	6.4	6.1	6.5	1	0	0	3 ”	6.0	5.8	6.3	6.0	0	0	0	
5/12	6.0	5.8	6.2	6.0	0	0	0	5 ”	stalled	stalled	stalled	—	—	—	—	
5/14	5.6	5.8	6.6	6.0	0	0	0	5 ”	6.2	6.4	6.9	6.5	0	0	0	210
5/15	6.3	6.0	6.1	6.1	0	0	0	5 ”	6.4	6.0	6.0	6.1	0	0	0	
5/16	7.2	6.1	6.1	6.4	0	0	0	5 ”	5.4	5.8	5.8	5.7	0	0	0	
5/17	6.9	6.0	6.4	6.4	0	0	0	5 ”	4.9	5.9	5.9	5.6	0	0	0	
5/18	6.2	6.0	5.7	6.0	0	0	0	5 ”	5.0	5.6	5.8	5.6	0	0	0	
5/19	6.0	6.4	6.3	6.2	0	0	0	5 ”	6.4	6.0	6.0	6.1	0	0	0	
								Cobra Venom.	One hour after administration							
6/12	6.4	6.4	6.4	6.4	0	0	0	.5 m.u.	6.0	6.1	5.8	6.0	0	0	0	210
6/13	6.9	6.8	5.8	6.5	0	0	0	.5 ”	6.0	5.6	6.0	5.9	0	0	0	
6/14	7.1	6.4	6.2	6.6	0	0	0	.5 ”	6.0	5.6	5.4	5.7	0	0	0	
6/15	6.0	6.0	6.0	6.0	0	0	0	.5 ”	6.0	5.6	5.8	5.8	0	0	0	
6/16	6.8	6.0	5.9	6.2	0	0	0	.5 ”	5.6	5.6	5.9	5.8	0	0	0	
6/18	6.0	5.8	5.8	5.9	0	0	0	.5 ”	5.8	5.8	5.5	5.7	0	0	0	
6/19	6.0	6.0	6.1	6.0	0	0	0	.5 ”	5.2	5.0	5.4	5.2	0	0	0	213
6/20	6.1	6.4	5.6	6.0	0	0	0	1 ”	5.8	5.4	5.5	5.6	0	0	0	
6/21	5.8	5.8	5.8	5.8	0	0	0	1 ”	5.0	5.8	5.4	5.4	0	0	0	
6/22	5.9	6.0	5.1	5.8	0	0	0	1 ”	4.8	5.3	5.2	5.1	0	0	0	
6/23	6.0	5.9	6.0	6.0	0	0	0	2 ”	5.9	5.8	5.9	5.9	0	0	0	212
									60.0+	60.0+	60.0+	two hours later				

depressed by the narcotic at first, they showed no such effect later. Similar series of daily injections of cobra venom, given the same animals after a period of rest, produced no symptoms of habituation.

10928 P

Behavior of Rats in a Maze in Relation to Analgesic Effect of Cobra Venom and Morphine.

MOSES B. MACHT. (Introduced by David I. Macht.)

From the Department of Psychology, Johns Hopkins University, and the Pharmacological Research Laboratory, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

An investigation of the effect of repeated injections of cobra venom and morphine on behavior of rats in a circular maze has been described in a previous paper,[1] where it was shown that such administration of morphine effected habituation or tolerance. No habituation, on the contrary, followed injections of cobra venom. Doses of 0.5 mouse unit effected a slight stimulation of the animals' behavior while the primary stimulation produced by larger doses of cobra venom, 1 to 2 mouse units, was followed by a secondary depression, from which the rats recovered the next day. Cobra venom as well as morphine now being used therapeutically for relief of pain, the writer wished to discover whether the analgesia produced by these drugs bore any relation to the general depression in behavior they effect. Eight rats were therefore selected from a series trained in the maze and their running time on 11 successive days was studied after injections of morphine and cobra venom, respectively. Pain threshold was also daily determined before and after administration of each drug. The method employed for studying analgesia has already been described[2] and the various pain thresholds were expressed quantitatively in volts. The results obtained are illustrated by Tables I and II, one showing the effect on running time and pain threshold of rats of successive injections of morphine and the other that of similar treatment with cobra venom. Difference in running time before and after injection of morphine and cobra venom is expressed in seconds and the pain threshold is set forth in volts. Minus signs prefacing figures for running time indicate stimulation or shortening of time of performance after drug's administration, as compared with rats' normal behavior.

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[1] Macht, M. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 433.

[2] Macht, D. I., and Macht, M. B., *J. Am. Pharm. A.*, 1939, in press.

TABLE I.
Relation Between the Running Time and Analgesia of Rat After Morphine.

Date (June, '39)	Milligrams of morphine injected	Average difference in running time sec	Difference in pain threshold, volts
12	3	stalled	368
13	3	stalled	430
14	3	60.0	260
15	3	+ 1.6	124
16	3	+ 0.8	89
18	5	60.0	279
19	3	+ 0.2	30
20	3	+ 0.3	32
21	3	— 1.0 (stimulation)	20
22	5	stalled	656
23	5	+ 2.6	172

TABLE II.
Relation Between Running Time and Analgesia of Rat After Cobra Venom.

Date (June, '39)	Mouse units of cobra venom injected	Average difference in running time, sec	Difference in pain threshold, volts
12	.5	— .2	30
13	.5	— .2	85
14	.5	— .6	45
15	.5	— .3	165
16	.5	— .1	25
18	.5	— .3	85
19	.5	— .6	75
20	.5	— .5	35
21	1.0	— .4	305
22	1.0	— .5	654
23	2.0	60.0	654

It is apparent that the analgesic action of morphine runs parallel to the general depression it effects. Where the morphine injections produced marked general depression of rat, there the greatest degree of analgesia was observed. When the rats had become habituated to the narcotic and their running time was but little impaired, however, the degree of analgesia was very small as indicated by the slight difference in pain thresholds.

In case of cobra venom the findings were radically different, there being no such analogy between general behavior of the rats in the maze and the degree of analgesia produced by the drug. Small doses of cobra venom, 0.5 mouse unit, stimulated activity of the animals and even effected a slight analgesia. Larger doses, 1 mouse unit, of cobra venom did not impair the running time but raised the pain threshold. It was only after a much larger dose of the venom, 2 mouse units or more, that depression of the animals' behavior accompanied a deep analgesia.

Such findings, made throughout the series, agree with clinical observations reported by various clinicians[3, 4, 5] and throw some light on the mechanism of analgesia produced by cobra venom as compared with that of morphine. The latest work[6] on the physiology of pain seems to point to the hypothalamus as that part of the brain most intimately concerned with sensations of pain. Cobra venom produces analgesia through its direct effect on the hypothalamus or some lower synapse while the action of morphine is not restricted to that region but spreads over the cortical areas, thus depressing the animals' general behavior.

Summary. A comparative study of behavior of rats in the maze and the analgesia produced in them by morphine reveals that the two effects run parallel, *i. e.,* the greater the general depression, the greater the analgesia. In case of cobra venom, however, analgesia or heightening of the pain threshold is produced without any marked effect on general behavior of the animals.

10929

Sugar Alcohols. XIX. Relationship between Chemical Constitution and Speed of Hemolysis.

ANNE MARY KUNKEL, C. JELLEFF CARR AND JOHN C. KRANTZ, JR.

From the Department of Pharmacology, School of Medicine, University of Maryland.

In recent studies[1] concerned with the diuretic action of isomannide (1, 4, 3, 6 dianhydromannitol) it was observed that this compound hemolyzed red cells through osmotic pressure phenomena. This observation prompted the study of the hemolytic activity of a number of sugar alcohols and their anhydrides on the red cells of the dog. The hemolytic activity of certain of these compounds, namely, glycerin, ethylene glycol, erythrol and mannitol has been reported previously by Jacobs.[2]

The apparatus described by Jacobs[3] was employed. The sugar alcohols and their anhydrides met the usual criteria of identity and purity as previously established by the authors. Solutions isosmotic with blood in recently boiled distilled water were prepared. The pH was determined by the glass electrode and the osmotic pressure was measured cryoscopically. Volumes of 20 cc of the solution were employed and 0.1 cc of freshly drawn defibrinated dog's blood supplied the red cells. The apparatus was standardized using water and saline solutions of various degrees of hypotonicity to establish the time hemolysis relationship. The time periods required for 90% hemolysis at 37°C are shown in Table I.

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[3] Macht, D. I., *Ann. Int. Med.,* 1938, **11**, 1824.

[4] Macht, D. I., *M. Press,* 1939, **201**, 254.

[5] Rutherford, R. N., *New England J. Med.,* 1939, **221**, 408.

[6] Propper-Graghtchenkoff, N. I., *Acta Medica U.S.S.R.,* 1938, **1**, 110.

[1] Krantz, J. C., Jr., and Carr, C. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 577.

[2] Jacobs, M. H., *J. Cell. and Comp. Physiol.,* 1933-34, **4**, 161.

[3] Jacobs, M. H., and Parpart, A. K., *Biol. Bull.,* 1931, **60**, 95.

Using red cells from various species the rates of hemolysis caused by mannitol and isomannide have been compared. These data are shown in Table II.

TABLE I.
Rates of Hemolysis by Sugar Alcohols and Anhydrides.

Compound	pH	Freezing point—(°C)	Avg time for Hemolysis (90%) 3 exp.
Methyl Alcohol	7.20	.53	9 sec
Ethylene Glycol	7.18	.51	4 ”
” Oxide	7.18	.55	5 ”
Glycerin	7.05	.54	4 ”
Epihydrin Alcohol	7.11	.54	10 ”
Erythrol	7.00	.56	120 min
Erythritan	6.90	.58	12 sec
Isosorbide	7.18	.60	23 ”
Penta Erythritol	7.20	.57	75 min
Arabitol	6.90	.56	75 ”
Mannitol	7.09	.57	>240 ”
Mannitan	7.18	.54	210 ”
Isomannide	7.02	.59	8 sec
Styracitol	6.90	.59	120 min
Sorbitol	7.12	.57	150 ”
Sorbitan	7.11	.58	135 ”
Polygalitol	6.92	.60	120 ”
Dulcitol	7.13	.54	>240 ”
Inositol	6.90	.60	60 ”
Pinitol	7.00	.55	70 ”

TABLE II.
Rate of Hemolysis of Red Cells of Different Species by Isotonic, Neutral Solutions of Isomannide and Mannitol.

Species	Isomannide, Seconds	Mannitol, hours
Cat	6	>4
Rat	7	>4
Guinea Pig	7	>4
Human	9	>4
Dog	8	>4
Monkey	16	>4
Rabbit	11	>4
Ox	14	>4

All of the hemolytic phenomena in Table I have been shown to be osmotic in character and not erythrolytic, as in each case the hemolysis is prevented by isotonic salt solution. To avoid the influence of saline constituents upon the speed of hemolysis unbuffered solutions were employed, hence the variations in pH of the solutions of the compounds studied. Jacobs[3] has stated that under certain conditions variations of 0.01 pH unit may produce a measurable effect upon the degree of hemolysis of red blood cells. In these experiments where effort was made to have the solute in isosmotic concentration, pH changes of much greater magnitude did not produce measurable changes in the speed of hemolysis. However, we may emphasize the necessity of considering the reported speeds of hemolysis as relative, dependent upon a great number of factors including individual variations among animals of the same species.

Summary. An inspection of the data shows that the sugar alcohols and their anhydrides up to 3 carbon atoms in the molecule rapidly hemolyze red cells. With erythritol containing 4 carbon atoms the hemolysis is slow, but that produced by its anhydride, erythritan, is rapid. The sugar alcohols containing 5 and 6 carbon atoms in the molecule and their monoanhydrides slowly hemolyze red cells. The double anhydrides, isomannide and isosorbide, cause rapid hemolysis. Mannitol hemolyzes the red cells of several species slowly; isomannide induces rapid hemolysis of the red cells of the same species.

10930

The Electroencephalogram of Schizophrenic Patients During Administration of Vitamin B_1.

MORTON A. RUBIN.

From the Memorial Foundation for Neuro-Endocrine Research, Worcester State Hospital, Worcester, Mass.

Recent studies[1] suggest that a deficient oxygen metabolism is present in schizophrenia. Increasing the rate of oxygen consumption in schizophrenics by means of thyroid[2] and dinitrophenol[3] has resulted in some clinical improvement. Since vitamin B_1 is known to influence oxygen consumption of the brain,[4] is seemed desirable to investigate its effect on the schizophrenic psychosis. The results of this study by Dr. L. H. Chase have been published elsewhere.[5] The present report has to do with the electroencephalograms recorded during the major investigation. It has been found previously that thyroid[6] and dinitrophenol[7] increase the frequency of the "alpha" rhythm of the electroencephalograms. If vitamin B_1 had the same effect, more direct evidence would be at hand to decide whether the medication influenced the metabolism of the brain.

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[1] Hoskins, R. G., *Arch. Neurol. and Psychiat.*, 1937, **38**, 1261; Rheingold, J. C., *Psychsom. Med.*, 1939, **1**, 397.

[2] Hoskins, R. G., and Sleeper, F. H., *Am. J. Psychiat.*, 1930, **87**, 411.

[3] Looney, J. M., and Hoskins, R. G., *Am. J. Psychiat.*, 1935, **91**, 1009.

[4] O'Brien, J. R., and Peters, R. A., *J. Physiol.*, 1935, **85**, 454.

Five schizophrenic patients were given 100 mg of synthetic crystalline vitamin B_1 daily* for a period of 6 to 9 weeks. The medication was given orally except for the last 2 weeks, when it was administered intravenously. Weekly electroencephalograms were obtained from each patient for 3 weeks prior to medication, during medication and for 3 weeks after discontinuation of vitamin B_1. Small, lead disc-electrodes were placed over the occipital lobes, and potentials recorded with reference to a pair of earthed leads on the mastoid processes. Two Grass amplifiers and ink-writing undulators were employed.

The "alpha" frequency remained within normal limits of variation[8] throughout the medication and post-medication periods. Likewise the amount of "alpha" activity *(percent time alpha)* did not vary more during medication than it did before or after vitamin B_1 administration.

Vitamin B_1 administration has no observable influence on brain potentials from normal pigeons, but does have an appreciable effect in avitaminotic pigeons.[9] Our failure to find changes in frequency or amount of "alpha" waves in schizophrenic patients, coupled with the findings in pigeons, leads to the conclusion that the schizophrenics studied do not have an avitaminosis B_1. In addition, it follows that vitamin B_1 is not involved in the decreased oxygen consumption reported for the schizophrenic patient.

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[5] Chase, L. H., *Am. J. Psychiat.*, 1939, **95**, 1035. No significant changes were found in blood pressure, pulse rate, oxygen consumption rate, venous carbon dioxide and glutathione levels.

[6] Rubin, M. A., Cohen, L. H., and Hoagland, H., *Endocrinol.*, 1937, **21**, 536.

[7] Hoagland, H., Rubin, M. A., and Cameron, D. E., *J. Neurophysiol.*, 1939, **2**, 170.

* We are indebted to Merck and Co. for their generosity in supplying us with synthetic crystalline Vitamin B_1 (Betabion).

[8] Rubin, M. A., *J. Psychol.*, 1938, **6**, 325.

[9] Tokaji, E., and Gerard, R. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 653.

10931

Effect of Parenteral Liver Extract on the Fission Rate of *Paramecium caudatum.*

Austin R. Middleton and George E. Wakerlin.

From the Department of Biology, University of Louisville, and the Department of Physiology, College of Medicine, University of Illinois.

The antipernicious anemia or hemopoietic liver principle possesses the well-known property of stimulating the production and maturation of red blood cells, leucocytes, and blood platelets. Consequently, we studied the effect of parenteral liver extract on the rate of fission of another type of cell; *viz., Paramecium caudatum.* If the fission rate of this organism were significantly increased by the liver principle, the possibility of utilizing the procedure as a bioassay method for the antipernicious anemia principle suggested itself.

The method of determining the fission rate of *Paramecium caudatum* was essentially that of Jennings[1] as modified by Middleton,[2] and involved the daily isolation of the organisms and the determination of their fission rates by direct observation at 24-hour intervals. The antipernicious anemia liver extract was used in the form of a parenteral liver extract labelled 3 cc derived from 100 g of liver and now labelled 10 units per 3 cc.* The culture medium used was a 0.05% solution of malted milk in distilled water. The fission rates of *Paramecium caudatum* were determined for periods ranging from 5 to 50 days in concentrations of liver extract in the malted milk solution, varying from 0.1 to 0.00005% on the basis of fresh liver equivalent. The minimum effective concentration in pernicious anemia patients is about 0.01% of liver equivalent. Suitable controls with liver extract inactivated by heating at 100°C for 24 hours and with the plain malted milk solution were employed. For each determination, 20 specimens of paramecium were employed.

The results outlined in Table I show that in all of the concentrations used, liver extract failed to produce a consistent significant increase in the fission rate of *Paramecium caudatum.* In the 6 highest concentrations of liver extract and in all of the concentrations of inactivated liver extract, there was a significant decrease in the fission rate. Statistical analysis of the results obtained during 5 successive

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[1] Jennings, H. S., *J. Exp. Zool.,* 1913, **14**, 279.

[2] Middleton, A. R., *J. Exp. Zool.,* 1915, **19**, 451.

* Supplied by Dr. Guy W. Clarke, Lederle Laboratories, Inc., Pearl River, New York.

TABLE I.
Effect of Active and Inactivated Parenteral Liver Extract on the Fission Rate of *Paramecium caudatum.**

		Avg No. of fissions per line for the 20 lines of each experiment			
Conc. in % of liver equivalent	No. of days obser-vation	Active liver extract	Heated liver extract	Malted milk controls	Excess† of controls
.1	5	2.1 ± .104		3.6 ± .074	1.5 ± .127
.05	5	1.8 ± .081		3.8 ± .108	2.0 ± .108
.025	5	3.2 ± .010		3.6 ± .111	0.4 ± .012
	16	11.3 ± .199		14.2 ± .098	2.9 ± .222
.013	16	12.0 ± .168		16.0 ± .162	4.0 ± .234
.006	16	13.0 ± .126		15.0 ± .137	2.0 ± .186
	10	9.0 ± .075		9.2 ± .060	0.2 ± .096
	16	14.6 ± .113		15.7 ± .113	1.1 ± .159
.003	50	35.3 ± .262		38.1 ± .340	2.8 ± .429
	65		34.3 ± .3986	48.6 ± .279	14.3 ± .487
	10	9.2 ± .060		8.3 ± .069	—0.9 ± .606
.0016	50	37.1 ± .318		38.1 ± .340	1.0 ± .466
	65		35.8 ± .552	48.6 ± .279	12.8 ± .736
	10	9.4 ± .719		8.3 ± .069	—1.1 ± .715
.0008	50	34.7 ± .373		31.7 ± .432	—3.0 ± .515
	65		35.9 ± .455	48.6 ± .279	12.7 ± .497
.0004	50	33.8 ± 1.272		31.7 ± .432	—2.1 ± .728
	65		35.8 ± .545	48.6 ± .279	12.8 ± .612
.00005	50	33.8 ± .561		31.7 ± .432	—2.1 ± .708
	65		37.3 ± .612	48.6 ± .279	11.3 ± .454

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* 3 cc derived from 100 g of liver.

† Differences which are statistically significant are underscored.

10-day periods of the 50- and 65-day observations with active and inactive liver extracts failed to reveal any progressive stimulation or retardation of the fission rate.

Conclusions. In the range of its effective concentration in the body of the pernicious anemia patient, parenteral liver extract does not significantly change the fission rate of *Paramecium caudatum.* In the higher concentrations employed, liver extract decreased the fission rate. Inactivated liver extract decreased the fission rate in all concentrations used. Obviously the procedure has no value as a bioassay method for the antipernicious anemia principle.

10932

Amino Acid Nitrogen in Urine of Children with the Nephrotic Syndrome Following Intravenous Amino Acids.

LEE E. FARR AND DOUGLAS A. MACFADYEN.
From the Hospital of the Rockefeller Institute for Medical Research, New York.

Recently Farr and MacFadyen[1] reported observations on the concentration of amino acids in the blood plasma of children with the nephrotic syndrome. They observed that while the disease persisted the concentrations were subnormal. Furthermore, nephrotic crises[1, 2] were ushered in with a sudden further fall in plasma amino acid concentration and clinical recovery from these crises was attended by a rapid rise to the precritical subnormal concentration. The close association of the clinical course of the disease with changes in the amino acid content of the blood plasma suggested the possibility of treating these patients, particularly during nephrotic crises, by giving amino acids intravenously. A prerequisite to this therapy was the determination of urinary loss of amino acid by *nephrotic* children following intravenous amino acid injections. The present study was designed to bear on this point.

Four children with the nephrotic syndrome on the wards of the hospital were chosen for study. Two were males, W. O'B. and J. C., 5 and 6 years of age respectively, and 2 were females, E. S. and R. Q., both 7 years of age. These children had all been studied extensively during the period of their hospitalization which varied from 7 to 14 months. At the time of the study all had characteristically low plasma proteins, normal hemoglobin, normal blood urea nitrogen, normal or elevated urea clearances, normal blood pressure and absence of hematuria. All were free from obvious acute or focal infection.

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[1] Farr, L. E., and MacFadyen, D. A., *Am. J. Dis. Child.*, in press.

[2] Farr, L. E., *Am. J. Dis. Child.*, in press.

Methods of analysis. Total urinary N.P.N. was estimated on trichloracetic acid urine filtrates by the gasometric micro-Kjeldahl method of Van Slyke.[3] Urine urea plus ammonia nitrogen by the gasometric technic of Van Slyke and Kugel,[4] and urinary amino acid nitrogen by the ninhydrin method of MacFadyen and Van Slyke.[5]

Procedures. After an overnight fast a urine specimen was obtained from each patient to empty the bladder. Two hundred cc of water were then given and urine specimens were obtained until the total quantity was sufficient to obviate small errors of collection. Immediately after this specimen was obtained 5.0 g of casein hydrolysate (generously furnished by Mead Johnson and Co.) were given intravenously in a 10% solution. Again urine specimens were obtained over the intervals ranging from 65 to 166 minutes immediately following this intravenous medication. At this juncture the patients were fed their usual breakfast. Urine was then collected throughout periods varying from 73 to 116 minutes following the meal.

TABLE I.

Simultaneous Determinations of Urinary Excretion Rates of Total Non-Protein Nitrogen, Urea Nitrogen and Amino Acid Nitrogen, Before and After Intravenous Acids and a Protein Meal in Nephrotic Children.

Patient, Age in yrs	Sex	Condition of patient	Time of period in min	Urine vol., cc/min	Excretion per min		
					N.P.N. mg	Urea + NH_3 N mg	Amino N mg
J.C.	M	Fasting	147	1.67	3.98	3.28	.042
		Amino acid I.V.*	166	1.15	3.34	2.77	.057
6		Breakfast[1]	116	0.95	3.19	2.77	.041
W.O'B.	M	Fasting	174	0.235	4.02	3.52	.034
		Amino acid I.V.*	162	0.327	4.68	3.81	.065
5		Breakfast[2]	99	0.403	3.86	3.28	.011
E.S.	F	Fasting	192	0.304	4.65	4.16	.031
		Amino acid I.V.*	65	0.831	5.75	4.62	.073
7		Breakfast[3]	73	0.452	5.38	4.41	.056
R.Q.	F	Fasting	200	0.325	4.98	4.35	.041
		Amino acid I.V.*	100	0.600	5.79	4.79	.071
7		Breakfast[4]	83	1.096	5.30	4.62	.053

* 5.0 g of casein hydrolysate (640 mg amino nitrogen).
[1] Protein 7 g, fat 15 g, carbohydrate 62 g.
[2] ” 11 ” ” 20 ” ” 75 ”
[3] ” 20 ” ” 30 ” ” 62 ”
[4] ” 23 ” ” 33 ” ” 77 ”

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[3] Van Slyke, D. D., *J. Biol. Chem.*, 1927, **81**, 235.

[4] Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, 1933, **102**, 489.

[5] MacFadyen, D. A., and Van Slyke, D. D., to be published.

Results. Table I shows the results obtained. In 3 of the 4 children the urinary excretion per minute of non-protein and urea nitrogen was slightly increased after the casein hydrolysate was given. In one child the values for these substances decreased. The excretion per minute of amino acid nitrogen increased slightly in each instance. Following the meal the loss of amino nitrogen was greater than the fasting level in 2 children, less in one and the same in one. In each child there was slightly less amino nitrogen in the urine after eating than after intravenous injection but the range was small.

Assuming that all the amino acid nitrogen secreted in the urine during the period immediately following intravenous injection was derived from the amino acid mixture given, the loss amounted to approximately 1%. Since normally there is some excretion of amino acids in the urine even under fasting conditions, it may be concluded that the loss of injected amino acids as such is insignificant. The absence of an increase in the excretion per minute of total non-protein nitrogen and urea plus ammonia nitrogen indicated that the amino acids given were not rapidly broken down to end-products of metabolism and excreted. This suggests that the nitrogen supplied by a casein hydrolysate given intravenously is available for nutritional needs. Further studies have already been carried out on this point and will be reported later.

Summary. There was no significant immediate increase in the urinary excretion per minute of non-protein nitrogen, urea plus ammonia nitrogen nor amino acid nitrogen, by 4 nephrotic children 5 to 7 years of age who had received intravenously 5 g of an amino acid mixture from casein hydrolyzed by enzymes. Therefore it appears probable that the injected amino acids are utilized for the nutritional needs of the patients.

10933 P

Effect of Pyocyanin on Cerebral Metabolism.*

J. F. Fazekas, Harriet Colyer, Sarah Nesin and H. E. Himwich.

From the Department of Physiology and Pharmacology, Albany Medical College, Union University, Albany, New York.

Young[1] has demonstrated that pyocyanin evokes an initial stimulatory effect on the respiratory exchange of excised cerebral tissue,

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* Aided by a grant from the Child Neurology Research (Friedsam Foundation).

[1] Young, L., *J. Biol. Chem.*, 1937, **120**, 659.

an effect which is subsequently followed by an irreversible depression. Gerard[2] has reported beneficial results with small concentrations of pyocyanin for the treatment of dementia praecox. The present communication consists of a report of results obtained with large concentrations of pyocyanin (5 to 10 times that used by Gerard) on cerebral metabolism *in vivo*.

The animals were divided in two groups. The members of the first were operated under either ether or nembutal. The skull of dogs was trephined, exposing the superior longitudinal sinus. The etherized animals were then permitted to recover from the anesthesia and were, therefore, studied without the complicating effects of a narcotic. Blood samples were drawn practically simultaneously from the superior longitudinal sinus and the femoral artery and analyzed for oxygen[3] and glucose.[4] The second group of animals was studied for neurological changes. These dogs and cats received varying amounts of pyocyanin on from 2 to 4 successive days.

In 14 observations after the intravenous injection of pyocyanin (150 mg) variable results were obtained in the oxygen differences between the blood entering and leaving the brain of dogs. In 7 of the 14 experiments there was a rise in the oxygen consumption while in the other 6 experiments a decrease was noted. In one instance there was no change. The results obtained on a dog (Table I) reveal that the oxygen A-V difference first increased and then diminished, only to be followed by another increase after a second injection of pyocyanin. In this experiment blood sugar fell. Blood flow measurements have not been made in these experiments. However, it is improbable that such large variations in the A-V differences may be attributed entirely to variations of blood flow. In 16 experiments the effect of pyocyanin on the blood sugar was studied in both cats and dogs. In 9 of these experiments injection of pyocyanin was followed by a definite fall in the blood sugar while in 7 experiments no change was observed.

In the second group (6 dogs and 10 cats) pyocyanin produced acute and chronic neurological changes. Four of these dogs succumbed with tonic clonic convulsions after receiving 150 mg of pyocyanin. Since the symptoms observed resemble those noted in cats, they will be described together. The cats received from 16 to 20 mg of pyocyanin intraperitoneally on 3 or more occasions. Within a few days these animals exhibited varying deviations from their usual

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[2] Gerard, R. W., *Arch. Neur. and Psychiat.*, 1938, **40**, 985.

[3] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[4] Hagedorn, H. E., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 46.

TABLE I.
Cerebral Oxygen Uptake After Pyocyanin.

Min. after injection of pyocyanin	A.-V. Difference, vol. %	Blood sugar, mg%		Remarks
		Arterial	Venous	
	9.15	90	79	Control
15	15.68	83	68	150 mg pyocyanin, clonic spasms, vomiting
30	11.70	81	65	Panting
43	9.52			”
58	5.77	74	66	
		54	48	150 mg pyocyanin
20	11.52	52	20	

behavior. In all instances marked muscular weakness was noted. Those animals that were able to walk at all sank to the floor after a few steps. The uncontrolled movements of the head resembled those of an animal with disturbance of vestibular apparatus. In 2 instances the animals gave evidence of a release of emotional mechanism. For example, the animals ran about in a frightened manner with tail hairs widely spread. Six of the 10 cats succumbed later, although they received no additional dosage of pyocyanin. In those animals that survived, the neurological changes persisted for 3 weeks and gradual signs of improvement were noted. Further studies in the biochemical and morphological changes of the brain are now in progress.

Conclusions. Pyocyanin administered *in vivo* produces an increased cerebral oxygen utilization which may be followed by a decrease. These changes are accompanied by symptoms of disturbed function of the central nervous system.

10934

The Filterability of Bacteria.

S. P. Kramer. (Introduced by J. H. Northrop.)

If one filters a 1:30,000 solution of night blue, a colloidal basic dye, through a siliceous filter' Berkefeld N, the filtrate is colorless.

If, now, we add a so-called "wetting agent" to the dye solution it is found that the dye passes the filter. A number of such agents have been tried, of which I give a partial list: Blood serum, bile, sodium taurocholate, glycocholic acid, turkey red oil, mucin, soap, castor oil soap, lecithin, sodium lauryl sulfate, etc.

Similarly, one can, by adding such agents before inoculation to nutrient broth cultures of bacteria which are non-filterable through siliceous filters when grown on broth without such agents, render the bacteria filterable. The following experiments will illustrate:

The experiments were made as follows: In each case the filter flask contained before sterilization 100 cc of the nutrient broth plus any addition which was used in the broth culture which was to be filtered, so that the filtrate passed directly into fresh, sterile material without further handling.

The ordinary laboratory meat broth is made by extracting ground lean beef in the cold room for 24 hours, then autoclaving at 120°C for 30 min, adding NaCl and peptone, and filtering and adjusting the pH to the required degree.

Berkefeld N filters were used throughout.

April 20, 1939. Ten different samples of an ordinary house meat broth culture of *Streptococcus hemolyticus* Group C were passed through 10 different Berkefeld N filters. All the filtrates showed growth of the streptococcus. The same organism grown in the Liebig meat extract broth were filtered through 10 different Berkefeld N filters. None of the filtrates showed growth.

June 6, 1939 A 4-day culture of *Streptococcus hemolyticus,* Group C, grown in meat extract broth containing 1:1000 taurocholate of sodium, was passed through 4 different filters.

June 8, 1939. Three of these filtrates were infected with streptococcus.

April 24, 1939. A 24-hour culture of typhoid bacillus (Rawlings) in meat extract broth was passed through 4 different Berkefeld filters.

April 25, 1939. All filtrates were sterile and there was then passed through the same filter an 11-day culture of typhoid bacillus in meat extract broth to which was added 1:5000 of sodium lauryl sulfate.

April 26, 1939. All filtrates were infected.

The above experiment was repeated with culture containing 1:2000 sodium taurocholate. All filtrates were infected.

June 4, 1939. A 48-hour culture of typhoid bacillus in meat extract broth was filtered through each of 4 different Berkefeld filters.

June 6, 1939. All filtrates were sterile. There was then passed through the same filters a culture made in another portion of the same broth which contained 1:1000 sodium taurocholate.

June 7, 1939. All filtrates were infected.

When filtrates were infected, the identity of the microörganism in the filtrates was established by stained smear culture, and also in the case of the typhoid bacillus, serologically.

April 11, 1939. A 24-hour culture of *Bacillus subtilis* was passed through each of 5 Berkefeld filters.

April 12, 1939. All filtrates were sterile. The filters were then sterilized in the autoclave and after cooling, there was passed through 3 of the filters a 2-day culture of *Bacillus subtilis* in broth containing 2:1000 sodium taurocholate. All these filtrates were infected the next day. Through the other 2 sterilized filters there was passed a broth culture of subtilis without any "wetting agent", and both of these filtrates remained sterile.

In the course of this work it was found that when control experiments were made with broth cultures grown on the ordinary laboratory meat broth without any added "wetting agent" a considerable percentage of the controls showed that the organisms had passed the filter. It was found that if the dye is added to this ordinary laboratory meat broth before heating it did not pass the filter. When added to the broth which had been autoclaved, the dye passed the filter.

Further, it was found that when the broth was made with Liebig meat extract (½%) the dye did not pass the filter. I finally reached the conclusion that in the case of broth made with ground beef there was always a considerable amount of fat present and the heating converted this into soap, and this small amount of soap present was the "wetting agent" which aided the dye and bacteria to permeate the filter.

When meat extract, in the preparation of which the temperature never goes above the boiling point of water, was used to make the culture medium, neither the dye nor bacteria passed the filter.

This was supported by experiments which showed that a solution of castor oil soap of 1:50,000 passed the dye through the Berkefeld filter.

Analysis of the 2 broth culture media by the Division of Chemistry, National Institute of Health, showed in the case of the one made from ground beef, 45 parts per million of fatty acid, and in the one made from meat extract 24 parts per million. It is not unlikely that Kendall[1] in his experiments with typhoid bacilli grown on his "K" medium, which he found to be filterable, had by accident one of these "wetting agents" present. His medium was prepared from the intestines of swine or sheep and autoclaved and it is entirely possible that he had present either soap or bile salts, or both.

As a working hypothesis to explain the action of the various wetting agents on filters, I believe the wetting agent "insulated" the surface of the filter pores.

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[1] Kendall, A. I., *Trans. Assn. Am. Physicians*, 1932, **57**, 100.

Siliceous filters have a negative charge and hold the basic dye particles which have a positive charge. When the filtering surface is covered with the "wetting agent" this prevents the siliceous surface from holding the positively charged dye particles.

This hypothesis is borne out by the following experiment. A clean glass rod was immersed in the dye solution in distilled water and allowed to stand over night. The rod will be found stained blue and the dye can not be washed off with water. Another glass rod is immersed in a dye solution containing 1:2000 castor oil soap, or a similar solution of sodium taurocholate, or a solution of sodium lauryl sulfate of similar concentration. The rods are allowed to stand in these solutions over night, yet when they are removed and washed off with water, they are unstained.

The "wetting agent" has insulated the surface of the glass rod, which has a negative charge, so that it can not hold the positively charged dye particles.

It is a pleasing duty to acknowledge my thanks to the staff of the National Institute of Health for the facilities placed at my disposal.

10935

Effect of Certain Endocrines on the Excretion of an Antidiuretic Substance.

STEVENS J. MARTIN AND HERMAN C. HERRLICH.

From the Department of Physiology and Pharmacology, Albany Medical College, Union University, Albany, N. Y.

The activity of the posterior pituitary has been investigated with renewed interest during the past few years. It has been shown[2] recently and confirmed[1, 4, 5] that an antidiuretic substance is excreted in the urine following dehydration of animals. Gilman and Goodman believe this substance is of pituitary origin since it is absent in the urine of animals dehydrated after hypophysectomy.

The relationship of the antidiuretic activity of the posterior pituitary to the other members of the endocrine system is still not clear. It has been reported that it is related to adrenal cortical function only

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[1] Boylston, G. A., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 644.

[2] Gilman, A., and Goodman, L. S., *J. Physiol.*, 1937, **90**, 113.

[4] Ingram, W. R., Ladd, L., and Benbow, J. T., *Proc. Am. Physiol. Soc.*, 1938, 107.

[5] Martin, S. J., Herrlich, H. C., and Fazekas, J. F., *Am. J. Physiol.*, 1939, **127**, 51.

in so far as they are both concerned with water metabolism.[5] In this investigation, an attempt has been made to determine the effect of chronic daily injections of certain sex hormones on the ability of the posterior pituitary to excrete an antidiuretic substance during dehydration.

A series of 84 adult albino and piebald rats were used in this study, 4 of which were females and were hypophysectomized, the remainder were males and were divided into groups for injections of endocrine preparations. The products employed* were oil solutions of theelin, testosterone propionate and progesterone and the gonadotropic principle of human pregnancy urine (Antuitrin S). All injections were made subcutaneously once or twice daily in the amount and for the duration noted in Table I. The concentrations were adjusted so that the daily injected amounts varied only from 0.1 cc to 0.3 cc. At the end of the experimental period, the hypophyses of 14 theelin- and 12 testosterone-treated rats were assayed for their gonadotropic activity by the method of intramuscular implantation into immature female rats.[6]

Throughout this study, the rats were kept in groups of 4 in cages constructed to facilitate urine collections. Twenty-four-hour urine samples were collected after periods of (1) free access to water, (2) 24-hour dehydration, and (3) 24-hour dehydration beginning 1-3 days after the injections of the endocrine preparations were stopped. The samples were bioassayed for the presence of an antidiuretic principle according to the technic modified by Gilman and Goodman. As in previous studies, the *index of diuresis* was regarded as the number of minutes required for a group of 4 hydrated rats to excrete one-half the amount of water given by stomach tube after a subcutaneous injection of the dialyzed, concentrated 24-hour urine sample. All rats were given a standard diet and the usual laboratory care.

Assays were also made on similar daily urine collections obtained from the 4 female rats intermittently on different days, during a 12-day period after hypophysectomy. Successful removal of the pituitary was shown by the post-operative absence of oestrus with the aid of daily vaginal smears as well as by the absence of pituitary tissue upon careful autopsy examination.

Results. The assays of urine obtained from all rats given free access to water showed an average index of diuresis of 109 minutes.

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* We wish to express our thanks for the generous supply of theelin and Antuitrin S from Dr. Oliver Kamm, Parke, Davis & Co., and of testosterone propionate and progesterone from Dr. Erwin Schwenk, Schering Corp.

[6] Meyer, R. K., Leonard, S. L., Hisaw, F. L., and Martin, S. J., *Endo.*, 1932, **16**, 655.

TABLE I.
Index of Diuresis Following Dehydration of Adult Rats After Chronic Injections of Endocrine Preparations.

Procedure	No. of animals	Daily dosage per rat	No. of days of injections	Total No. of assays	Index of Diuresis avg in min	Remarks
Control	84	—	—	21	166 (147-201)	Before injections
Injections of:						
Theelin	24	200 i.u.	26	12	180 (148-255)	After cessation of injections
Progesterone	12	2 mg	10	13	157 (134-209)	
Testosterone propionate	24	1 mg	24	12	154 (136-195)	
Antuitrin S	12	20 i.u.	23	10	164 (138-210)	

This value was normal and indicated the absence of an antidiuretic substance. When the animals were dehydrated for 24 hours, the average index was 166 minutes, showing that an antidiuretic principle was present in the urine. In 8 tests on the dehydrated hypophysectomized rats, the index of diuresis was 104.5 minutes (range 92-116 minutes) and was therefore within normal values.

The effect of chronic daily injections of large doses of various endocrine preparations on the excretion of an antidiuretic substance after dehydration is presented in Table I. A total of 58 bioassays were performed. All rats showed a normal appetite throughout this study. It will be noted that the group averages for the indices of diuresis (154-180 minutes) cover a fairly wide range. However, in each determination, except one not recorded in the table, the index was significantly greater than the normal control value and hence definitely indicated the presence of an antidiuretic substance in the urine. The ability of the pituitary to respond to the stimulus of dehydration was not diminished by chronic injections of the endocrines used. That theelin and testosterone injections had some effect on the pituitary was shown by a decrease in gonad stimulating power of the anterior lobe of 43 and 35% respectively as determined by differences in the ovarian weights of immature rats.

It appears fairly well established that an antidiuretic substance is excreted in the urine as a result of dehydration whether this be from simple water deprivation[1, 2, 4] or hypoadrenia.[5] Whether such a substance is solely of pituitary origin may remain open to question until adequate chemical methods of identification replace the bioassay procedure now used. However, the significant fact still remains that an antidiuretic principle is not present in the urine of dehydrated animals after hypophysectomy. This finding of Gilman and Goodman[2] is confirmed by our results.

It has been previously shown that large doses or prolonged injections of theelin[6] or of testosterone propionate[3, 7] depress the gonad-stimulating power of the anterior hypophysis. While we have noted this, our results also show that these preparations do not affect the posterior lobe in its ability to secrete an antidiuretic substance following dehydration. Furthermore, progesterone and antuitrin S similarly have no effect. In one respect, therefore, the sex hormones are not directly related to posterior pituitary activity. Indirect relationships, of course, may be possible.

Conclusions. Prolonged injections of large doses of theelin, progesterone, testosterone propionate and antuitrin S do not alter the ability of dehydrated rats to excrete an antidiuretic substance. Since this substance appears to be of hypophyseal origin, these sex hormones have no direct relationship to this phase of posterior pituitary activity.

10936

Effects of a Digested Pituitary Extract on Reproductive Tract of Hypophysectomized Adult Male Rats.

R. O. GREEP.

From the Squibb Institute for Medical Research, New Brunswick, N. J

The work of McShan and Meyer[1] and Chen and van Dyke[2] has shown that the luteinizing ability of pituitary gonadotropic extracts can be largely destroyed by tryptic digestion. These treated preparations produced remarkably specific gametogenic effects in normal and hypophysectomized immature female and male rats.

Greep and Fevold[3] have shown that the hypophyseal fraction LH acts on the interstitial cells of hypophysectomized adult male rats causing the secretion of male hormone, whereas the FSH preparation will sustain or repair the gametogenic processes without stimulating the testes to endocrine function. However, with dosages of FSH in excess of the minimal requirements for normal tubular function slight but definite growth of the accessories occurred. Since the hypophysectomized adult male rat offers quite a satisfactory test animal for determining the "purity" of the FSH fraction, it, therefore, seemed of interest to investigate its response to a digested pituitary extract in which the luteinizing action had been largely abolished.

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[3] Hamilton, J. B., and Wolfe, J. M., *Endo.*, 1938, **22**, 360.

[7] Moore, C. R., and Price, D., *Endo.*, 1937, **21**, 313.

[1] McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1938, **126**, 361.

[2] Chen, G., and van Dyke, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 172.

[3] Greep, R. O., and Fevold, H. L., *Endocrinology*, 1937, **21**, 611.

Digestion* of the original (horse) pituitary extract was carried out in a manner identical with that employed by Chen and van Dyke.[2] The injections were made once daily from the 10th to the 20th postoperative day with necropsy on the day following the last injection. The effects of the digested and of the control non-digested extracts on the weight of the testes and seminal vesicles are shown in Table I. The non-digested extract produced marked growth of the testes and the seminal vesicles attained a larger size even than those of normal animals of comparable weight. Histologically the testes resembled those of normal adult rats except for an increased amount of interstitial tissue (Fig. 1, 2).

The digested material though less active in stimulating testicular growth did nevertheless, with adequate dosage, produce considerable enlargement. The spermatogenic processes, interrupted by 10 days total hypophyseal deficiency (Fig. 3), were restored to normal by the trypsin digests given in daily doses of 1 and 4 mg (Fig. 5, 6) but not in daily doses of 0.2 mg (Fig. 4). Spermatozoa were abundant in the epididymides and motility was normal when the two higher doses were administered. The atrophic interstitial cells remained unchanged (Fig. 3, 6), although in some animals the dose of digested extract was 4 times as great as that which clearly maintained

TABLE I.

Material	Daily dosage, mg powder	No. animals	Testes, mg	Empty seminal vesicles, mg
Non-digested pituitary powder	0.2	2	2349	656
Digested pituitary powder	0.2	2	864	84.4
	1.0	2	1210	100.8
	4.0	3	2035	111.9
Hypophysectomized	10 days	2	1795	104
,,	20 ,,	2	855	102
Unoperated controls		3	2603	343.3

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* The enzyme used (Merck's trypsin) was of the same preparation as that employed by Chen and van Dyke.[2]

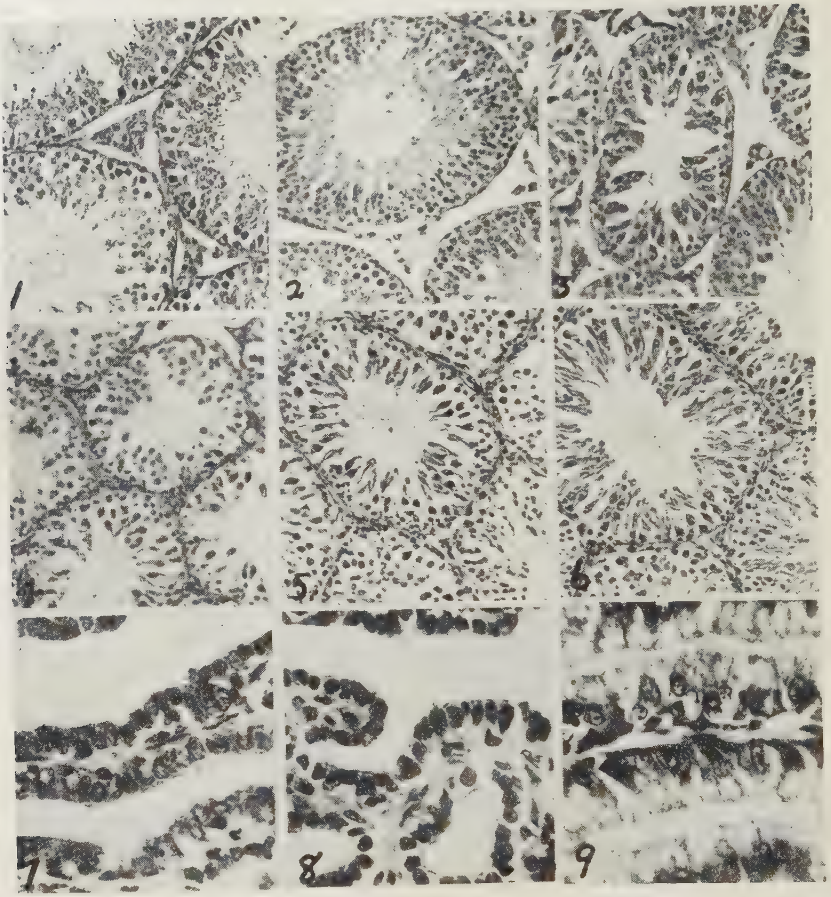

Fig. 1-6 (all ×120) are photomicrographs of adult rat testes; all injections were made on days 10-20 after hypophysectomy. 1. Operated rat receiving 0.2 mg non-digested pituitary powder daily. 2. Normal rat. 3. Untreated rat 10 days after hypophysectomy. 4, 5, and 6, hypophysectomized rats receiving 0.2, 1, and 4 mg digested pituitary powder daily respectively.

Fig. 7-9 (all ×540) show the epithelium of the seminal vesicles 10 days after operation (7), after 10 days treatment with 4 mg digested pituitary extract daily (8), and after similar treatment with 0.2 mg non-digested extract daily (9).

spermatogenesis. The weights of the seminal vesicles showed a total absence of any growth effect; microscopic examination also failed to reveal any stimulation of the glandular epithelium (compare Fig. 7, 8, 9). The scrotal area of these animals also remained in such an atrophic state that the gonads were restrained in the upper portion of the inguinal canal. Whatever effect this position may have on the testicular response to FSH presumably would be unfavorable.

Another experiment in which an extract of sheep pituitary glands was digested by this same enzyme preparation has yielded results which are similar to those just described.

Conclusions. Digestion of an anterior pituitary extract by trypsin under suitable conditions may destroy interstitial-cell stimulating activity, leaving a pure gametogenic effect in adult hypophysectomized male rats. It appears that interstitial-cell stimulation or luteinization is caused by the same substance which is largely destroyed by the digestion.

10937

Effect of Induced Hypercholesterolemia on Antibody-Response in Rabbits.

MAURICE R. CHASSIN AND MAURICE BRUGER.

From the Department of Medicine, New York Post-Graduate Medical School and Hospital, Columbia University, New York.

Although it is generally assumed that cholesterol in blood and tissue is related in some manner to processes of immunity,[1, 2] the mechanism of this action is not clearly understood. It seemed important to determine whether the production of antibodies by the organism was influenced by the level of cholesterol in the blood.

Seventeen rabbits (9 controls and 8 experimental) varying in age from 3 to 5 months were fed Ralston-Purina rabbit-chow once daily. Hypercholesterolemia was produced in the experimental group by feeding 1 g of cholesterol mixed with the food 3 times a week for a period of 5 to 8 weeks. After this time, the blood-cholesterol usually exceeded 350 mg per 100 cc and immunization was then begun. The feeding of cholesterol to the experimental group was continued throughout the experiment.

All animals received intravenous injections of increasing amounts of a typhoid vaccine containing 10^9 organisms in 1 cc. Injections of 0.2, 0.3, 0.4, 0.6, 0.8, 0.9, and 1.0 cc were made on the 1st and 5th, 8th, 11th, 14th, 16th, 19th, and 21st day respectively.

Agglutinative tests were done before injections of vaccine and at weekly intervals for 5 weeks thereafter. The titer was that dilution which, after refrigeration overnight and after careful agitation, still showed macroscopic clumping. The results are given in Table I.

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[1] Muller, G. L., *Medicine,* 1930, **9**, 119.

[2] Stoesser, A. V., and McQuarrie, I., *Am. J. Dis. Child.,* 1935, **49**, 658.

TABLE I.

Rabbit No.	Weeks											
	0		1		2		3		4		5	
	Titer	Chol.	Titer	Chol.	Titer	Chol.	Titer	Chol.	Titer	Chol.	Titer	Chol.
Control Group												
28	0	114	1:40	117	1:160	165	1:320	170	1:1280	162	1:1280	155
29	0	99	1:40	110	1:40	98	1:160	92	1:320	100	1:320	96
30	0	92	1:40	89	1:320	101	1:160	121	1:640	110	1:640	108
26	0	85	1:40	73	1:160	100	1:320	110	1:320	75	1:320	101
27	0	110	1:40	116	1:80	115	1:160	83	1:320	85	1:320	96
51	0	86	1:40	89	1:640	108	1:1280	131	1:1280	119	1:640	120
53	0	85	1:40	87	1:1280	121	1:1280	116	1:2560	123	1:2560	130
54	0	79	1:40	79	1:1280	94	1:2560	110	1:1280	104	1:1280	110
56	0	90	1:160	88	1:320	107	1:1280	108	1:2560	151	1:2560	140
Avg	0	93	1:55	94	1:475	112	1:835	115	1:1175	114	1:1100	117
Experimental Group												
31	0	407	1:320	721	1:2560	1000	1:2560	1154	1:1280	986	1:1280	1415
33	0	390	0	750	1:1280	986	1:5120	949	1:5120	950	1:2560	1390
36	0	421	0	417	1:1280	600	1:5120	694	1:2560	773	1:2560	1173
37	0	535	0	475	1:1280	637	1:10240	620	1:5120	675	1:5120	1014
25	0	789	1:80	841	1:640	883	1:5120	927	1:2560		1:1280	603
27a	0	417	1:160	514	1:320	547	1:1280	522	1:2560	525	1:2560	475
28a	0	852	1:160	721	1:320	803	1:2560	798	1:2560	882	1:1280	791
29a	0	357	1:80	475	1:160	529	1:640	682	1:640	556	1:640	568
Avg	0	521	1:100	614	1:980	748	1:4080	793	1:2800	764	1:2160	929

Blood-cholesterol (modified Sackett's method[3, 4]) is expressed in mg per 100 cc of whole blood.

Conclusion. Following injection of rabbits with typhoid vaccine, a higher agglutinin-titer against *E. typhosa* was obtained in those with induced hypercholesterolemia† as compared with control rabbits having normal blood cholesterol.

10938 P

Macromolecular Components of Untreated and of Formolized Normal Chick Embryo Tissue.*

D. GORDON SHARP, A. R. TAYLOR, HAROLD FINKELSTEIN AND J. W. BEARD.

From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

Macromolecular substances have been found in healthy tissues of both plants and animals.[1] In experiments associated with studies of the equine encephalomyelitis virus protein[2] and the non-infectious immunizing principle[3] of formalin-treated virus-diseased chick embryos,[4] we have obtained such substances from normal chick tissue and from the tissue treated with formalin.

Chick embryos incubated for 11 days were harvested, chilled, the heads removed and the bodies ground in a Ten Broeck grinder held in cracked ice. The resulting body mince was made to a 20% suspension and extracted as indicated. Large particles were removed with the angle centrifuge and the resulting extracts fractionated by

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[3] Sackett, G. E., *J. Biol. Chem.*, 1925, **64**, 203.

[4] Mirsky, I. A., and Bruger, M., *J. Lab. and Clin. Med.*, 1932, **18**, 304.

† Because cholesterol, *per se*, in increased concentration in the blood might produce false agglutination, the sera of 3 rabbits with marked hypercholesterolemia (700 to 800 mg per 100 cc) produced by the feeding of cholesterol were tested; no clumping was observed.

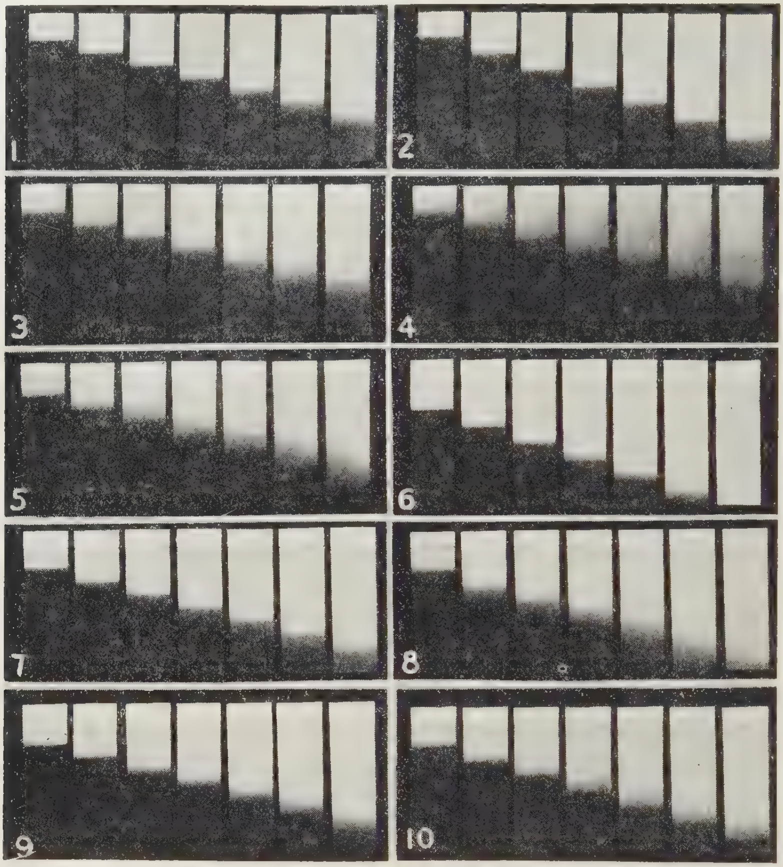
* This work was supported by a grant from Lederle Laboratories, Pearl River, N. Y.

[1] Wyckoff, R. W. G., *Cold Spring Harbor Symposia on Quantitative Biology*, 1938, **6**, 361.

[2] Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 771.

[3] Beard, J. W., Finkelstein, H., Sealy, W. C., and Wyckoff, R. W. G., *Science*, 1938, **87**, 89.

[4] Beard, J. W., Finkelstein, H., Sealy, W. C., and Wyckoff, R. W. G., *Science*, 1938, **87**, 490.



Sedimentation diagrams of macromolecular components from extracts of untreated normal embryo tissue and from the tissue treated with formalin. The ultracentrifugal field employed to obtain the photographs was about 47,000 g and the interval between exposures was 5 minutes.

Fig. 1. Normal untreated tissue extracted in dilute buffer. Pellets dissolved in buffer and photographed after 2 ultracentrifugal cycles.

Fig. 2. Same preparation after 3 cycles showing boundary of increased sharpness.

Fig. 3. Normal untreated tissue extracted with 0.9% saline. Pellets of second cycle dissolved in saline.

Fig. 4. Same preparation after a third cycle. Note homogeneity not increased.

Fig. 5. Formolized tissue extracted with water and pellets dissolved in dilute buffer. Photographed after the second cycle.

Fig. 6. Same preparation after third ultracentrifugal cycle.

Fig. 7. Formolized tissue extracted with 0.9% sodium chloride solution. Second cycle.

Fig. 8. Same preparation after the third cycle. The boundary is more diffuse than after the second cycle.

Fig. 9. Untreated tissue extracted with water and pellets dissolved in water. Second cycle.

Fig. 10. Same preparation after third cycle.

alternate ultracentrifugal runs of 9,000 g for 5 minutes and 67,000 g for 1 to 1½ hours. The products have been studied by ultracentrifugal analysis.

The results observed when extraction was made and the pellets were dissolved with 0.005 M ammonium hydroxide-ammonium chloride buffer pH 8.2 are shown in Figures 1 and 2. After 2 ultracentrifugal cycles, double or diffuse boundaries were present whereas only one of slight diffuseness was seen after the third cycle. Here $s_{20}^{\circ} =$ ca 70×10^{-13} cm sec^{-1} dynes^{-1}. A similar material was obtained by extraction with glycerin in 50% concentration in the dilute buffer. When extraction was made in 0.9% sodium chloride solution alone or combined with glycerin and the pellets were dissolved in saline, indications of the same conponent were evident after the second cycle (Fig. 3) but further homogeneity was not attained by additional cycles (Fig. 4). The sharpest boundaries were obtained with material derived by extraction of tissue and solution of the pellets in distilled water (Fig. 9 and 10).

A component with sedimentation pattern indistinguishable from that of the derivative from untreated tissue was obtained from normal tissue to which formalin was added to 0.4% (as in the preparation of chick vaccines[4]). When formalin was added to tissue suspended in water, relatively sharp boundaries with little spread were obtained with the pellets dissolved in dilute phosphate buffer (0.005 M, pH 7.0) (Fig. 5 and 6), and the sedimentation constant was $s_{20}^{\circ} =$ ca 70×10^{-13}. Extraction, formolization and fractionation in 0.9% sodium chloride solution gave a similar product of much less homogeneity (Fig. 7 and 8). The observed sedimentation constant varied somewhat under both conditions, depending on protein concentration, but $s_{20}^{\circ} =$ ca 70×10^{-13} was always observed with proper pellet dilution and was maintained with increased dilution to extinction of the boundary.

These results demonstrate the presence of a macromolecular protein or a group of such substances of related size in normal chick embryo tissue, persisting in a similar or but slightly altered form on treatment of the tissue with dilute formalin

10939 P

Macromolecular Components of Chick Embryo Tissue Diseased With the Virus of Equine Encephalomyelitis.*

A. R. Taylor, D. Gordon Sharp, Harold Finkelstein and J. W. Beard.

From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

Chick embryo tissue diseased with virus of equine encephalomyelitis (Eastern strain) has been studied in relation to the homogeneous macromolecular substances recently obtained from normal tissue[1] and the results observed are described here.

Eleven-day chick embryos were inoculated with virus and harvested when moribund. The heads were removed and the body tissue was ground without diluent in chilled Ten Broeck grinders. Extraction, ultracentrifugal fractionation and analysis were similar to those of the previous studies.[1]

When the diseased tissue mince was extracted in 20% suspension with a mixture of equal parts 0.9% sodium chloride solution and glycerin at pH 8.5 and the pellets were dissolved in 0.9% saline (the sequence employed by Wyckoff[2] for isolation of the equine encephalomyelitis virus protein[3]) the component with $s_{20}^{\circ} = \text{ca } 250 \times 10^{-13}$ cm sec^{-1} dynes^{-1} was observed in the second or third ultracentrifugal cycle (Fig. 1 and 2). This product was infectious to the order of 10^{14} mouse units per gram, and the result corroborated previous findings.[3]

On the other hand, when extraction was made with water, no evidence of this material was seen; instead, only boundaries with $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$ cm sec^{-1}dynes^{-1} were obtained (Fig. 3) as from non-diseased tissue.[1] Infectivity to the order 10^{14} was likewise associated with this product.

Fractionation of 0.9% saline extracts of virus-diseased tissue formolized to 0.4% yielded pellets which, on solution in saline, gave diffuse boundaries indicative of $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$. Formolization and extraction with water at pH 7.0 gave a product with relatively sharp boundaries (Fig. 3). These derivatives of 0.9% saline and of

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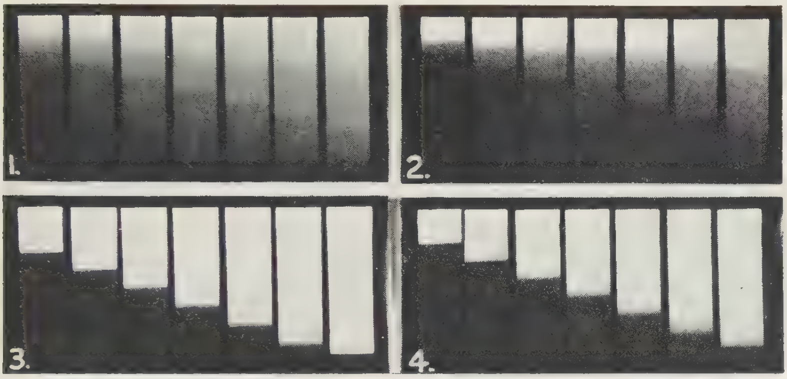
* This work was aided by a grant from Lederle Laboratories, Pearl River, N. Y.

[1] Sharp, D. G., Taylor, A. R., Finkelstein, H., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 459.

[2] Wyckoff, R. W. G., personal communication.

[3] Wyckoff, R. W. G., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 771.



Sedimentation diagrams of ultracentrifugal fractions of virus-diseased chick embryo tissue.

FIG. 1. Product from diseased tissue extracted in mixture of equal parts 0.9% sodium chloride solution and glycerin. Photographed at 3-minute intervals in an ultracentrifugal field of 17,000 g after third fractionation cycle. $s_{20}^{\circ} = \text{ca } 250 \times 10^{-13}$ cm sec.$^{-1}$ dynes^{-1}.

FIG. 2. A similar highly infectious preparation showing the light, diffuse boundary indicative of the normal tissue component $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$ cm sec.$^{-1}$ dynes^{-1}.

FIG. 3. Material from water extract of diseased tissue photographed at 5-minute intervals in a field of 47,000 g. $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$ cm sec.$^{-1}$ dynes^{-1}. Infectious titre 10^{14} mouse units per gram.

FIG. 4. Component from water extract of formolized diseased tissue photographed in field of 47,000 g. $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$ cm sec.$^{-1}$ dynes^{-1}.

water extraction were both highly antigenic, protecting guinea pigs against 1000 mouse infectious units of virus given intracerebrally. Thus far this material from formolized virus-diseased tissue is indistinguishable by ultracentrifugal analysis from the non-immunizing product from formalin-treated normal tissue.[1]

The product $s_{20}^{\circ} = \text{ca } 250 \times 10^{-13}$ has not been obtained from non-diseased tissue.[1] Extracts of low salt content from both diseased and normal tissue yield products similar in quantity and sedimentation pattern ($s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$) that are apparently identical and unrelated to $s_{20}^{\circ} = \text{ca } 250 \times 10^{-13}$. Unstable in solutions of salt concentration necessary for demonstration of $s_{20}^{\circ} = \text{ca } 250 \times 10^{-13}$, the presence of this normal component is indicated only in the very diffuse pattern associated with $s_{20}^{\circ} = \text{ca } 250 \times 10^{-13}$ (Fig. 2). Further work is necessary to explain the high infectivity of $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$ from diseased tissue, though this may be due to the presence of quantities of $s_{20}^{\circ} = \text{ca } 250 \times 10^{-13}$ too small for ultracentrifugal detection.

The presence of $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$ in formalin-treated normal tissue as well as formalin-treated virus-diseased tissue raises the question of the significance of the component previously described as

$s_{20}^{\circ} = \text{ca } 60 \times 10^{-13}$ associated with the immunizing principle.[4] Although $s_{20}^{\circ} = \text{ca } 70 \times 10^{-13}$ from formolized diseased tissue is consistently highly antigenic, the actual immunizing principle involved may be unrelated to and only non-specifically associated with it.

10940 P

Glucuronic Acid Produced by Surviving Slices of Liver from Animals Poisoned with Phosphorus or Chloroform.

ERNEST BUEDING* AND PETER LADEWIG. (Introduced by W. Goldfarb.)

From the Biochemical Institute and the Institute of Experimental Pathology, University of Istanbul, Turkey.

It has been previously demonstrated[1] that surviving liver slices shaken in the Warburg apparatus in a saline phosphate medium (pH :7.4) produce conjugated glucuronic acids in presence of borneol, menthol or phenol. This production is significantly increased by the addition of sodium-lactate or pyruvate. The present investigation is concerned with the glucuronic acid synthesis by liver slices excised from guinea pigs poisoned with phosphorus or chloroform. Phosphorus poisoning was produced by the subcutaneous injection of 0.75 mg of yellow phosphorus (dissolved in olive oil) per 100 g animal weight. Other guinea pigs were injected with 0.03 to 0.035 cc of chloroform per 100 g animal weight on 2 successive days, and the livers were removed 3 days after the first injection. The glucuronic acid production of slices of these livers was determined with and without addition of sodium-lactate. The method was described in previous publications.[1]

As a result of phosphorus poisoning the addition to liver slices of lactate produced no significant increase in either the oxygen uptake or the glucuronic acid production. Histologically these livers showed fatty infiltration, swelling of the nuclei and, in the terminal stages of the poisoning, diffuse nuclear destructions and no signs of regeneration. The addition of lactate to liver slices poisoned with chloroform resulted in an increase of the oxygen uptake and of the

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[4] Beard, J. W., Finkelstein, H., Sealy, W. C., and Wyckoff, R. W. G., *Science*, 1938, **87**, 89.

* Psychiatric Division, Bellevue Hospital, New York City.

[1] Lipschitz, W., and Bueding, E., *Proc. XVI Internat. Physiol. Cong.*, Zurich, 1938, 120; *J. Biol. Chem.*, 1939, **129**, 333.

TABLE I.
Liver Slices of Guinea Pigs Poisoned with Phosphorus or Chloroform.

		Q_{O_2}		Mg glucuronic acid produced by 1 g of dried liver	
		Saline containing .01% borneol	Saline containing .01% borneol and .02 M lactate	Saline containing .01% borneol	Saline containing .01% borneol and .02 M lactate
20 normal controls	Limits	3.5-5.6	5.9-8.6	1.1-5.4	1.7-11.4
	Avg	4.8	6.9 (+44%)	2.85	5.05 (+77%)
10 guinea pigs poisoned with phosphorus	Limits	2.8-5.0	2.65-7.0	0.25-3.8	0.25-4.9
	Avg	4.05	4.7 (+16%)	1.8	2.0 (+11%)
9 guinea pigs poisoned with chloroform	Limits	3.9-8.8	6.4-10.7	3.0-6.95	5.4-11.2
	Avg	6.2	8.25 (+33%)	4.5	7.6 (+69%)

The figures in parenthesis show the percentage increases over normal.

glucuronic acid production, the percentage increase being equal to that observed in normal liver slices. Histological examination of these livers showed, in addition to fatty infiltration, a large number of hypertrophied nuclei and mitoses, signs of marked regeneration. A series of guinea pigs injected with the same doses of chloroform and which were not sacrificed for the experiments died spontaneously 3 or 4 days after the first injection. Their livers showed the same signs of regeneration. Animals observed for 2 days after the first chloroform injection showed no regeneration.

Conclusions. The mechanism of the glucuronic acid production of liver slices is disturbed during phosphorus poisoning. On the other hand, in the terminal stages of fatal poisoning by chloroform the glucuronic acid production is not disturbed since the addition of lactate produced a normal increase of glucuronic acid. This was associated with histological evidences of regeneration.

10941

Mechanism of Pituitary Gonadotropic Antagonism.

Sibylle Tolksdorf and H. Jensen.

From the Biochemical Laboratory of the Squibb Institute for Medical Research, New Brunswick, N. J.

The capacity of certain pituitary extracts to act as gonadotropic antagonists upon intraperitoneal administration has been the object of several investigations.[1] We have recently shown, in collaboration with Dr. H. M. Evans and Dr. M. E. Simpson, that the antagonistic effect is due to the presence of the interstitial cell stimulating or luteinizing factor (ICSH, LH) in the pituitary preparation.[2, 3] We, therefore, assumed that the antagonistic factor is identical with the ICSH (LH). Fevold and Fiske have come to similar conclusions.[4] The mechanism and physiological significance of the gonadotropic antagonism is, however, still a matter of debate.

Recent investigations carried out in this laboratory indicate that the pituitary ICSH (LH) may be identical with the thyrotropic factor of the anterior pituitary since attempts to separate the two physiological effects by chemical means have so far been unsuccessful. This finding suggests that the secretion of the thyroid may be involved in the antagonistic action of the ICSH. It has been reported by various investigators[5, 6, 7] that the secretion of the thyroid inhibits the action of the follicle stimulating hormone (FSH) of the anterior pituitary. In order to correlate further the hypophyseal antagonistic effect with the secretion of the thyroid, we studied the influence of thyroxine on the ovarian response of certain gonadotropic principles under experimental conditions similar to those used in our investigations on the ICSH antagonism.

Experimental. Twenty-one to 23-day-old normal rats, weighing from 35 to 50 g were made hyperthyroid by the administration of varying amounts of thyroxine. As gonadotropic agents we used a purified pregnant mare serum preparation and a pituitary FSH. The ovarian response of the hyperthyroid animals to these gonadotropic factors was diminished in all instances as compared to the ovarian response of normal animals, 5 to 6 rats being used in each group.

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[1] Van Dyke, H. B., *The Physiology and Pharmacology of the Pituitary Body*, University of Chicago Press, Chicago, Ill., Vol. 2, 1939.

[2] Jensen, H., Simpson, M. E., Tolksdorf, S., and Evans, H. M., *Endocrin.*, 1939, **25**, 57.

[3] Evans, H. M., Simpson, M. E., Tolksdorf, S., and Jensen, H., *Endocrin.*, 1939, **25**, 529.

[4] Fevold, H. L., and Fiske, V. M., *Endocrin.*, 1939, **24**, 828.

[5] Smith, P. E., and Engle, E. T., *Anat. Rec.*, 1930, **45**, 278.

[6] Fluhmann, C. F., *Am. J. Physiol.*, 1934, **108**, 498.

[7] Leonard, S. L., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 599.

The inhibitory action of thyroxine on the effect of a gonadotropic preparation was demonstrated (1) upon simultaneous injection of both factors for 3 days either separately or mixed *in vitro*, and (2) upon treatment with thyroxine for 3 days previous to the administration of FSH. Table I gives an example of a 3-day test where thyroxine and pregnant mare serum were mixed *in vitro* and injected intraperitoneally, resulting in a definite reduction of the ovarian weights. Table II illustrates the antagonistic effect of thyroxine against hypophyseal FSH produced by subsequent administration of thyroxine and FSH, both given subcutaneously. A total dose of 0.05 mg of thyroxine was sufficient to diminish follicular development and to inhibit formation of an estrous uterus. Similar effects were observed when the thyroxine treatment was extended over a period of 8 days previous to the injection of FSH. It may be mentioned here that the inhibitory effect of thyroxine was observed on both subcutaneous and intraperitoneal injections while pituitary antagonism exerts itself only upon intraperitoneal administration.

The findings recorded in this paper are in agreement with results of similar experiments reported by different investigators. Fluh-

TABLE I.

Ovarian Response to Intraperitoneal Injection of Thyroxine and Pregnant Mare Serum, Mixed *in Vitro*. Autopsy performed 72 hours after onset of injection.

Thyroxine total dose, mg	Thyroxine alone, intrap., mg	PMS alone, intrap., mg	Thyroxine + PMS, mg	Controls, mg
0.4	9.8 SF	130 LF, CL	59 LF, CL	12 SF
0.2	—	130 LF, CL	74 LF, CL	—

Abbreviations: PMS, pregnant mare serum preparation (total dose 1 mg); SF, small follicles; LF, large follicles; CL, corpora lutea.

TABLE II.

Ovarian Response to Successive Subcutaneous Injection of Thyroxine and FSH.* All autopsies performed on day 7.

Thyroxine total dose, mg	Thyroxine alone day 1-3, mg	FSH* alone day 4-6, mg	Thyroxine day 1-3 + FSH day 4-6, mg	Controls mg
0.2	17 SF	43 MF	24 SF	17 SF
0.05	19 SF	—	21 S, MF	—

* Total dose 0.2 mg = 8 RU. SF, small follicles; MF, medium sized follicles.

mann[6] has shown that thyroid feeding decreases the effectiveness of pituitary gonadotropic preparations in rats. On the other hand, thyroidectomy will increase the effectiveness of hypophyseal gonadotropic fractions, but will not augment the response of pregnant mare serum,* chorionic gonadotrophin† and gamone.‡ [6, 8, 9] Tyndale and Levin[10] have examined the ovarian response of FSH preparations from menopause urine in normal, hypophysectomized and thyroxine-treated hypophysectomized rats. They observed that the physiological response of their FSH preparation is more limited in normal than in hypophysectomized rats for subluteinizing doses and believe this to be due to an inhibitory effect of the thyroid on follicular development. They also reported that administration of thyroxine to hypophysectomized rats reduces the response to menopause FSH.

The observations recorded indicate that the thyroid secretion can act as a physiological antagonist to follicular development. FSH activity is inhibited by thyroxine in normal rats (Table II) and in hypophysectomized rats (Tyndale and Levine). FSH activity is enhanced in the absence of the thyroid secretion, *i. e.*, in hypophysectomized and in thyroidectomized rats. Gonadotropic pituitary preparations having an effect on the thyroid, will elicit a greater ovarian response in thyroidectomized animals.[6] Gonadotropic preparations having no appreciable effect on the thyroid such as pregnant mare serum, gamone and the pregnancy urine principle, give the same ovarian response after thyroidectomy.[6, 9] In agreement with this latter observation is the finding that chorionic gonadotrophin, gamone and prospermin§ also fail to act as antagonists when given intraperitoneally in combination with pregnant mare serum. Unfractionated anterior pituitary extracts and hypophyseal ICSH are potent antagonists under these conditions.[3]

If the conception is correct that the secretion of the thyroid is involved in the mechanism of antagonism the inhibitory effect of ICSH should be less pronounced in thyroidectomized animals. Preliminary experiments in thyroidectomized rats‖ have shown that a combination of ICSH and FSH, given intraperitoneally, does not produce the same degree of antagonism in the operated animals as in the controls.

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* Containing predominantly LH and some FSH.

† Containing LH only.

‡ A preparation from pooled samples of menopause urine containing LH and FSH.

[8] Leonard, S. L., and Hansen, I. B.. *Anat. Rec.*, 1936, **64**, 203.

[9] Leonard, S. L., and DeFrates, J., *Endocrin.*, in press.

[10] Tyndale, H. H., and Levin, L., *Am. J. Physiol.*, 1937, **120**, 486.

§ A preparation from pooled samples of normal male urine, containing LH and traces of FSH.

‖ We are greatly indebted to Dr. J. DeFrates of the Endocrine Laboratory for performing the thyroidectomy on immature rats for us.

It should be pointed out that the antagonism of hypophyseal ICSH and thyroxine has so far been demonstrated in the immature rat only. We have injected, intraperitoneally, a combination of pregnant mare serum and ICSH into adult female rats with established cycles and were unable to produce any appreciable antagonism. The question whether gonadotropic antagonism can be observed in immature or mature male rats has not yet been studied.

Brief reference should also be made to the observations of Fevold, Hisaw and Greep[11] and Fevold and Fiske[4] who have studied the effect of estrogen treatment on the response to hypophyseal FSH. They find the FSH activity enhanced after short treatment with estrogen but inhibited after prolonged treatment. They explain their findings by the outpouring of varying amounts of LH from the pituitary under the stimulus of estrogen administration. Their observation that normal ovaries implanted into estrogen-treated animals fail to respond to FSH administration may be explained in the light of our assumption that the luteinizing factor stimulates the thyroid, *i. e.,* estrogen-treated animals may be considered to be in a hyperthyroid state.

It has been reported[3] that purified FSH preparations also show antagonism when given intraperitoneally in combination with pregnant mare serum. This phenomenon is, however, observed only in normal rats while the antagonism of hypophyseal ICSH against pregnant mare serum can be obtained in hypophysectomized as well as in normal rats. It is probable that the antagonistic effect observed with FSH is due to the formation and liberation of estrogen which in turn acts on the pituitary. In order to confirm this possibility, we have given α-estradiol¶ simultaneously with pregnant mare serum to normal rats in the 72-hour test and observed a significant reduction in the response of pregnant mare serum.

Conclusions. The observation that the secretion of the thyroid exerts an antagonistic effect on the ovarian response of certain gonadotropic principles agrees with our assumption of the identity of the thyrotropic factor with the pituitary interstitial cell stimulating hormone (ICSH or LH) and suggests a possible explanation for the mechanism of the antagonistic properties of the ICSH.

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[11] Fevold, H. L., Hisaw, F. L., and Greep, R., *Am. J. Physiol.,* 1935, **114**, 508.

¶ We are greatly indebted to Dr. E. Schwenk of the Schering Corporation for supplying us with this material.

10942

Gonadotropic Activity of Ant. Pituitary of the Finback Whale.

H. Jensen, E. M. K. Geiling and Sibylle Tolksdorf.

From the Squibb Institute for Medical Research, New Brunswick, N. J., and the Department of Pharmacology, University of Chicago, Ill.

The gonadotropic activity of anterior whale pituitary has been determined by Valsö[1] and by Wallen-Lawrence.[2] Using normal immature female rats, these investigators demonstrated the presence of the follicle-stimulating (FSH) and luteinizing (LH) factors. We have repeated these standardizations in hypophysectomized as well as normal rats, testing the whale pituitary samples at the same time for their ability to repair the ovarian interstitial tissue (ICSH) in hypophysectomized female rats and to act as a gonadotropic antagonist. As shown recently,[3, 4] these 2 properties are characteristic of the hypophyseal LH factor.

Three different samples of acetone-dried anterior whale pituitary and 40% alcohol extracts thereof[3] were tested at different dose levels in normal and hypophysectomized rats in the routine manner, giving one injection daily for 3 days with autopsy 72 hours after onset of therapy. The first sample injected either subcutaneously or intraperitoneally into hypophysectomized rats, at dose levels ranging from 2 mg to 0.25 mg, produced only follicular development. The M.E.D. was found to be 0.25 mg. No repair of the interstitial tissue was observed at any dose level between 2 mg and 0.25 mg and no pronounced antagonism was manifested when given at these dose levels intraperitoneally to normal rats in combination with pregnant mare serum.* The simultaneous absence of the interstitial stimulating and antagonistic effects of this preparation agrees with our assumption of their identity.

On the other hand, 40% alcohol extracts of samples II and III which had been collected later were found to contain both the FSH and LH (ICSH) factors and to give varying degrees of antagonism

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[1] Valsö, J., *Klin. Wochenschr.*, 1934, **13**, 1819; *Hvalradets Skrifter Norske Videnskaps—Akad. Oslo*, 1938, **16**, 5 (*Chem. Ab.*, 1938, **32**, 6707).

[2] Geiling, E. M. K., *Bull. Johns Hopkins Hosp.*, 1935, **57**, 123.

[3] Jensen, H., Simpson, M. E., Tolksdorf, S., and Evans, H. M., *Endocrin.*, 1939, **25**, 57.

[4] Evans, H. M., Simpson, M. E., Tolksdorf, S., and Jensen, H., *Endocrin.*, 1939, **25**, 529.

* It has been found[4] that purified FSH preparations may also act as an antagonist against the gonadotropic potency of pregnant mare serum.

TABLE I.
40% Alcohol Extract of Anterior Whale Pituitary, Sample II. Ovarian Response in 72-hr Test after Intraperitoneal Injection.

Hypophysectomized Rats 2 mg	Normal Rats 2 mg + PMS*	Normal Rats PMS* alone
9.2 mg slight foll. development, hypertrophy of interstitial tissue	71 mg (avg 6 rats)	119 mg (avg 6 rats)

* PMS: 1 mg total dose of a pregnant mare serum preparation.

when tested against pregnant mare serum (Table I). The gonadotropic activity of preparations II and III corresponds to that of similar preparations obtained from sheep and pig pituitary. Sample II (40% alcohol extract) when tested at 10 mg produced luteinization of the ovaries and stimulation of the thyroids and adrenals in hypophysectomized rats. In addition, 40% alcohol extracts of samples II and III were tested for thyrotropic activity in day-old chicks according to the method described by Smelser[5] and showed, at 4 and 2 mg total dose, approximately the same potency as similar extracts from sheep pituitary.

We are unable to give an explanation for the different results obtained with various whale pituitary samples. It is possible that in sample I the LH (ICSH) factor was destroyed by autolysis during the time which elapsed between the killing of the animal and the removal of the gland. In order to test this possibility, we studied the effect of autolysis on the gonadotropic content of anterior beef and whole sheep pituitary. The fresh glands were incubated at 37°C for periods varying from 2 to 6 hours and for 24 hours. Our assays of the incubated material are, however, not very conclusive and are, therefore, not given in detail. It seems that 6 hours' incubation causes a considerable reduction of the FSH and ICSH activities while incubation for 24 hours destroys practically all gonadotropic potency. We are under the impression that both factors are inactivated at approximately the same rate and that there is no sufficient differential destruction to allow a separation of the 2 factors. It might be desirable to study the rate of inactivation at incubation periods between 6 to 24 hours.

Summary. Three samples of acetone dried whale anterior pituitary and 40% alcohol extracts thereof were assayed in normal and hypophysectomized female rats. In the first sample, only the presence of FSH could be demonstrated, no interstitial cell stimulating, luteinizing, antagonistic or thyrotropic activity was observed at the dose

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[5] Smelser, G. K., *Endocrin.*, 1938, **23**, 429.

levels employed. Samples II and III showed the presence of both FSH and ICSH, concurrently with luteinizing, antagonistic and thyrotropic activity. Sample II was also tested for adrenotropic activity and it was found that this factor was present. The gonadotropic activity of 40% alcohol extracts of samples II and III is of the same order as that of similar preparations obtained from sheep and pig pituitary. The absence or presence of the multiple properties of the LH (ICSH) factor agrees with our assumption that they are due to one principle only.

10943

Alkyl Nitrites V. The Pharmacology of the High Molecular Weight Alkyl Nitrites.*

JOHN C. KRANTZ, JR., C. JELLEFF CARR AND SYLVAN E. FORMAN.
From the Department of Pharmacology, School of Medicine, University of Maryland.

In previous studies[1] the pharmacology of a series of new brominated and non-brominated alkyl nitrites of relatively high molecular weight was described. The properties and action of 2-ethyl-n-hexyl-1-nitrite[2] prompted a more complete investigation of this compound, which resulted in its use as a therapeutic agent in angiospastic disease. Cash and Dunstan[3] many years ago studied the activity of the primary and secondary alkyl nitrites from methyl to amyl. In general, they found that the higher nitrites, butyl and amyl, gave a greater fall in blood pressure than those of lower molecular weight. On the other hand, they found a greater duration of response when the lower molecular weight esters were administered. These investigators expressed the view that the magnitude of the fall in blood pressure was a function of the instability of the higher members and not of their molecular weight. The greater duration of the response of the lower members was attributed to their greater degree of stability. It oc-

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* The expense of this investigation was defrayed in part by a grant from the Committee on Therapeutics of the Council on Pharmacy and Chemistry of the American Medical Association.

[1] Krantz, J. C., Jr., Carr, C. J., and Forman, S. E., *J. Pharmacol. and Exp. Therap.*, 1938, **64**, 298.

[2] Krantz, J. C., Jr., Carr, C. J., and Forman, S. E., *J. Pharmacol. and Exp. Therap.*, 1938, **64**, 302.

[3] Cash, J. Th., and Dunstan, W. R., *Phil. Trans. Roy. Soc.*, 1893, **184** B, 505.

curred to the authors to extend this investigation further to include the nitrites of the higher molecular weight alcohols.

Compounds Studied and Methods. The method of synthesis and many of the constants of these compounds will be reported in a suitable journal. In the main, the compounds were odoriferous liquids with odors resembling the alcohols from which they were prepared. The assays were conducted by the nitrometer method set forth in the tenth revision of the United States Pharmacopoeia.

The vapor pressures were measured at 28°C in a Van Slyke apparatus.

Under nembutal anesthesia in the dog, the drugs were administered in 0.3 cc doses per animal. The ester was placed in a small aspirating bottle and was drawn into the trachea after volatilization. Table I shows the effect of this series of drugs on blood pressure.

Fig. 1 shows the relationship between the number of carbon atoms in the molecule of the alkyl nitrite, its vapor pressure and depressor response.

The graph shows that the depressor activity of the alkyl nitrites of high molecular weight is a function of their vapor pressures when administered by inhalation. The minimal effective vapor pressure of 0.5 mm is exhibited by n-decyl nitrite. Higher molecular weight compounds with equal vapor pressures are devoid of pharmacologic response when administered by inhalation. However, when these substances are dissolved in a hydroalcoholic solvent and injected intravenously they elicit full depressor responses. Branched chain compounds appear to be more active than those of the same molecular weight with straight chains. The former compounds exhibit a higher vapor pressure.

TABLE I.
Summary of the Effect of Alkyl Nitrites, 0.3 cc, on the Blood Pressure of the Dog.

Compound	No. of C atoms in molecule	Purity, %	No. of exper.	Original bl. p., mm Hg	Max. fall bl. p. %
n-hexyl nitrite	6	94	6	133	58
n-heptyl ”	7	93	7	126	47
n-octyl ”	8	99	3	151	30
2-et-n-hexyl-1 nitrite	8	92	5	130	46
n-nonyl nitrite	9	89	14	135	21
n-decyl ”	10	93	4	123	16
5-et-n-nonyl-2-nitrite	11	85	5	129	4
n-duodecyl nitrite	12	93	4	125	0
7-et-2-me-undecyl-1-nitrite	14	78	3	132	5
n-tetradecyl nitrite	14	85	4	136	0
n-hexadecyl ”	16	88	3	120	0
n-octadecyl ”	18	99	4	130	0

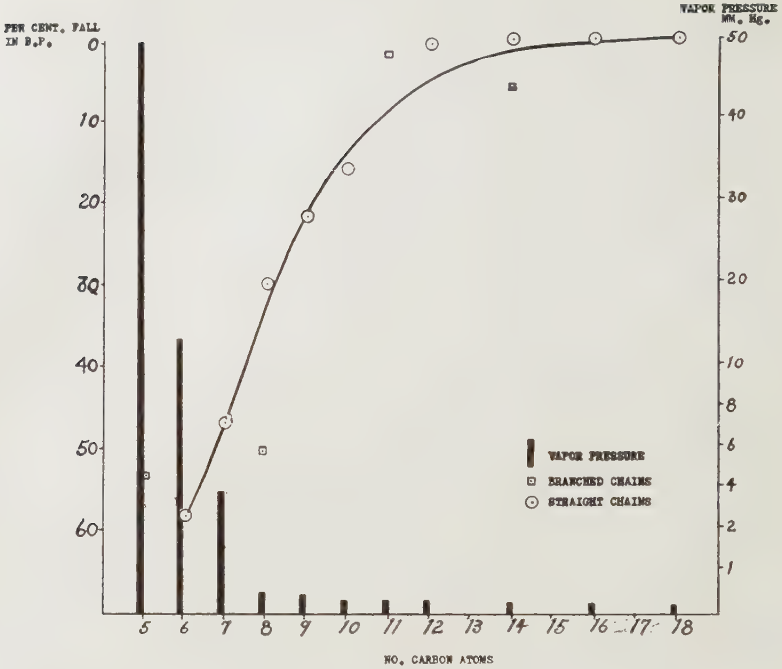

Fig. 1.

Blood Pressure, Vapor Pressure and Molecular Weight of Alkyl Nitrites.

These present studies do not agree entirely with the findings of Cash and Dunstan. First,[1] we have shown previously that ethyl nitrite gives as great a fall in blood pressure as does amyl nitrite. Second,[2] it was further established by studies on the speed of hydrolysis of amyl nitrite and 2-ethyl-n-hexyl-l-nitrite, that although their hydrolytic rates were practically identical, the higher molecular compound produced a depressor response lasting seven times as long as that elicited by the amyl ester. The authors attribute this to the lesser degree of water solubility of the 2-ethyl-n-hexyl-l-nitrite and its corresponding greater degree of oil solubility.

The vapor pressure of the compounds of this series reaches an optimum between 3 and 4 mm; higher vapor pressures give rise to compounds which produce an unnecessary plethora of methemoglobin in the animal's blood and, owing to a low oil solubility, exert a response which is ephemeral.

Conclusions. 1. The pharmacology of the higher alkyl nitrites has been investigated. 2. The relationship between molecular weight, vapor pressure and depressor response is demonstrated. 3. Certain theoretical considerations of the desiderata in physical properties necessary for depressor response are discussed.

10944

Comparison of the Influence of Anoxia and Asphyxia on Blood Sugar.*

E. GELLHORN AND A. PACKER.

From the Departments of Physiology and Psychiatry, University of Illinois College of Medicine.

In a preceding investigation, Gellhorn and Lambert[1] showed that the effect of a mixture of low oxygen and excess carbon dioxide is more effective in raising the blood pressure than would be expected from an algebraic summation of the effects of the 2 gases when studied separately. It was observed that this potentiating effect was present not only in the normal animal but also in dogs deprived of their buffer nerves. It seemed to us of interest to investigate whether a similar synergism could be observed with regard to the blood sugar effect. The investigations of Kellaway[2] have shown that in the unanesthetized cat low oxygen increased the blood sugar considerably, whereas the inhalation of carbon dioxide alone has no such effect. The mixture of the 2 gases was, however, not tested.

Eighteen experiments were carried out on 9 unanesthetized rabbits. Each experiment consisted of 3 observations obtained at one week intervals in which the effect of the inhalation of 7% O_2, 5.4% CO_2, and 7% O_2 plus 5.4% CO_2 respectively was studied for a period of 90 minutes. It was found invariably that the inhalation of 5.4% CO_2 had no significant effect on the blood sugar of rabbits. As Table I shows, the effects of 7% O_2 are slight and vary widely in different experiments. It was found, however, almost without exception, that when the low oxygen mixture was inhaled together with excess carbon dioxide, the blood sugar rose considerably higher than was observed in the same animal when subjected to low oxygen alone. The average rise in the blood sugar was 8.1% after the inhalation of 7% O_2 but 29.4% after the inhalation of 7% O_2 + 5.4% CO_2. An example of the individual records may be found in Fig. 1. It will be noted that the gas mixture low in O_2 and rich in CO_2 not only increased the blood sugar considerably more than the algebraic summation of the effects of the 2 factors alone but also that the

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* This work has been aided by a grant from the John and Mary Markle Foundation.

[1] Gellhorn, E., and Lambert, E. H., *The Vasomotor System in Anoxia and Asphyxia*, The University of Illinois Press, Urbana, 1939.

[2] Kellaway, C. H., *J. Phys.*, 1919, **53**, 211.

TABLE I.
Percentage Rise of Blood Sugar After 1.5 Hours.

A 7% O	B 7% O + 5.4% CO_2
+10	+64
+26	+28
+12	+64
— 2	+41
+ 4	+30
+ 3	+ 4
+ 2	+19
+ 1	+ 6
± 0	+43
— 7	+ 1
+28	+39
+ 7	+38
+16	+17
+17	+35
+ 9	+25
— 3	+22
+21	+42
+ 2	+11
Avg + 8.1	Avg +29.4

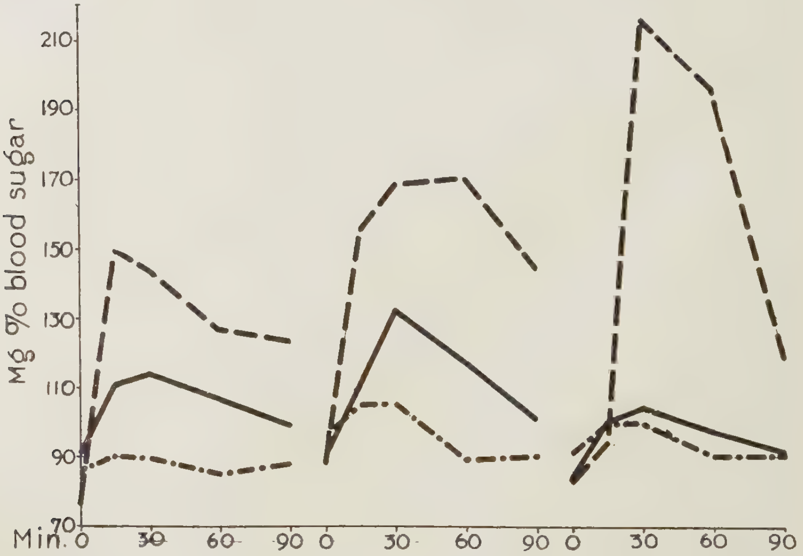

FIG. 1.

The influence of CO_2 and oxygen lack on the blood sugar of rabbits. The gases were administered from Douglas bags for 90 minutes.

Abscissa: Time in minutes.

Ordinate: Blood sugar in mg%.

———— During inhalation of 7% O_2.

—.—.—.—. " " " 5.4% CO_2.

— — — — " " " 7% O_2 + 5.4% CO_2.

effect of the combination of gases is more prolonged than is observed in experiments in which each of the gases alone was used.

Summary. Experiments on unanesthetized rabbits show that although the inhalation of 5.4% CO_2 has practically no effect on the blood sugar when used alone, it greatly increases the effect of the inhalation of 7% O_2 on the blood sugar.

10945

Effect of Season (Temperature) on Blood Lipids.*

O. B. Houchin and C. W. Turner.

From the Department of Dairy Husbandry, University of Missouri, Columbia, Mo.

In a previous paper[1] it was shown that an AP extract injected into New Zealand White female rabbits caused a significant depression of the plasma fat as determined by the Allen[2] method. Both the normal animals and the normal preinjection samples of blood from experimental animals showed rather large variations in plasma fat. Casual observations indicated that the lipid changes might be due to temperature changes accompanying the various seasons of the year. Further examination of our data indicated a rather definite drop in the plasma fat values during the increasingly cool weather in the fall, whereas the highest values appeared to be obtained during the hottest weather of the summer. On the other hand, previous studies on the seasonal variation in the blood lipids of the cow[2] and rabbit[3] did not indicate significant trends. The animals included in these studies were probably under fairly uniform temperature control in northern summer climate and housed during winter time. Recent study with the guinea pig[4] indicates an increased use of fat for maintenance under cold environment which condition might be expected to reduce the level of the blood lipids. Yet in the presence of an increased demand for fat, it would be possible for the blood lipids to remain high if the underlying mechanism regulating lipid metabolism was effective.

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* This study has been aided in part by grants from the Committee on Research in Endocrinology of the National Research Council. Contribution from the Missouri Agricultural Experiment Station, Journal Series, No. 630.

[1] Houchin, O. B., and Turner, C. W., *Endocrinology*, 1939, **24**, 638.

[2] Allen, B. N., *Minn. Agr. Exp. Sta. Bul.*, No. 130, 1938.

[3] Boyd, E. M., *Can. J. Res.*, 1938, **16**, 31.

[4] Kayser, C., *Compt. Rend. Soc. de biol.*, 1939, **126**, 701.

The object of this paper is to present a tabulation of our plasma lipid data according to season, in order to show the influence of temperature change with season upon blood lipids.

In this study as in the previous study, only mature New Zealand White female rabbits were used. Samples of ear blood were centrifuged and the plasma fat determined by Allen's method. During part of the experiment, the animals were kept in a temperature regulated room at between 75° and 80°F. At other times (as indicated) they were housed in outdoor cages subject to the environmental temperature.

When the rabbits were kept in a temperature regulated room at about 80°F the average plasma fat remained rather constant, but as the temperature both indoors and outdoors rose an increase in plasma fat was noted (Table I). As the weather became cooler, especially at night, the average plasma fat fell precipitously, reaching a minimum during the period of freezing temperatures. During the spring, the plasma fat again increased, although not markedly until both the day and night temperatures remained high.

These data are taken to indicate that there is a direct relation between environmental temperature and plasma fat in the rabbit. With high temperatures the blood lipids increase to extraordinarily high levels whereas in a cold environment (freezing), the lipids fall to low levels. On a functional basis it might be thought that the

TABLE I.
Seasonal Variation in Plasma Blood Fat of Rabbit.

			Plasma Blood Fat			
				Range		
Month	Environment	No. of Analyses	Avg mg%	High mg%	Low mg%	Remarks on Temperature or weather
March	Indoors	2	220	240	200	Constant 75°-80°F.
April	”	15	285	410	190	” ”
May	”	5	388	540	250	Temp. frequently above 90°F
June	Outdoors	14	384	830	200	Hot, cages not shaded
July	”	9	314	450	260	Very hot, cages shaded
Late August- Early Sept.	”	5	333	390	285	Warm days, cool nights
October	”	11	208	330	110	Nights cool
November	”	4	109	140	85	Frost at night
December	”	7	74	105	45	Cold—freezing temp. at night
January	”	8	43	70	25	Cold
February	”	14	36	55	22	Cold—temp. to 0°F
March	”	5	52	70	25	Warmer with great variation
April	”	5	71	170	40	Cool at night, changeable
May, early	”	7	71	140	40	” ” ” ”
May, later	”	11	205	400	100	Becoming very warm
June	”	10	250	420	140	Warm with some hot days

explanation of these observations rests upon the increased utilization of blood fat as a source of energy although it is not an uncommon physiological phenomena for increased utilization of a blood constituent to stimulate an increased rate of mobilization.

It is possible that environmental temperature may influence the rate of secretion (or level in pituitary) of the fat metabolism hormone and in turn influence the plasma fat level. It is planned to investigate this possibility.

From these data it is obvious that animals to be used for the assay of the fat metabolism hormone should be kept in a temperature-regulated room to avoid fluctuations in plasma lipid due to temperature.

10946

Lactogen Content of Pituitary of Pregnant and Lactating Rabbits and Guinea Pigs.*

SKAT HOLST AND C. W. TURNER.

From the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Columbia.†

Continuing the study of the lactogen content of the pituitary, the present paper presents our observations concerning the amount of lactogenic hormone in the anterior pituitary glands (AP) of female New Zealand White rabbits and guinea pigs in stages of pregnancy and lactation. The pituitaries, thyroids, adrenals and ovaries of normal animals sacrificed at various stages of pregnancy and lactation were removed and weighed. Each group of pituitaries was kept frozen until assayed with 20 pigeons by the Reece-Turner[1] method. Both pituitary gland weight and the assays are based upon the whole gland rather than the anterior lobe.

The pituitaries of the 10- and 20-day pregnant rabbits showed no increase of lactogen over the pituitaries from non-pregnant mature female rabbits whereas the pituitaries from the animals sacrificed 28 days after conception or just before parturition showed a slight increase from about 11 to 14 B.U. per pituitary (Table I).

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*This study has been aided in part by a grant from the Committee on Research in Endocrinology of the National Research Council.

† Journal Series No. 628.

[1] Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.*, 266, 1937.

TABLE I.
Lactogen Content of Pituitaries from Rabbits.

Stage of pregnancy or lactation	No. of animals	Avg body wt, g	Avg pituitary wt, mg	B.U. per pituitary gland, avg	B.U. per mg pituitary tissue, avg	B.U. per 100 g body wt, avg
10 days pregnant	4	2936	36.25	11.13	.307	.38
20 ,, ,,	5	3070	34.85	10.63	.305	.35
28 ,, ,,	5	3360	33.30	14.10	.423	.42
48 hr postpartum, young removed 15 hr	4	3067	31.90	18.33	.576	.60
48 hr postpartum, young removed 12 hr, then allowed 3 hr nursing	4	3146	54.61	15.31	.280	.49
5 days postpartum, young removed 15 hr	4	3105	44.35	40.31	.909	1.30
5 days postpartum, young removed 12 hr, then allowed 3 hr nursing	4	3270	49.15	32.19	.655	.98
10 days postpartum	4	3343	78.88	40.00	.507	1.20
20 ,, ,,	4	3403	59.68	26.63	.446	.78
30 ,, ,,	4	3465	56.55	15.75	.280	.45

TABLE II.
Lactogen Content of Pituitaries from Guinea Pigs.

Stage of pregnancy or lactation	No. of animals	Avg body wt, g	Avg pituitary wt, mg	B.U. per pituitary gland, avg	B.U. per mg pituitary tissue, avg	B.U. per 100 g body wt, avg
15 days pregnant	4	561	15.40	8.50	.552	1.52
30 ,, ,,	4	578	16.29	9.38	.576	1.62
45 ,, ,,	4	607	19.54	9.13	.467	1.50
58 ,, ,,	5	616	19.30	10.38	.538	1.68
48 hr postpartum, young removed 15 hr	4	518	17.06	18.38	1.077	3.55
48 hr postpartum, young removed 12 hr, then allowed 3 hr nursing	4	511	17.83	13.13	.736	2.57
10 days postpartum	4	566	19.26	15.19	.788	2.68
20 ,, ,,	4	540	18.43	12.45	.676	2.31
30 ,, ,,	4	561	18.08	9.63	.533	1.71

Two groups of animals were sacrificed 48 hours postpartum. In one group the young were removed 15 hours before the sacrifice of the mother and in the other group the young were removed for 12 hours and then returned for a 3-hour suckling period immediately before the sacrifice of the mother. This procedure was followed in an attempt to determine the influence of the stimulation of nursing on the lactogen content of the pituitary gland. The first group was found to contain 18.38 B.U. per pituitary or 0.576 B.U. per mg pituitary tissue while the second group contained 15.31 B.U. per pituitary or 0.280 B.U. per mg pituitary tissue.

Later it was found that the lactogen content of the AP had not reached its peak after 48 hours, therefore, 2 similar groups were obtained at 5 days postpartum. In this case the lactogen content in the group where the young had been removed the last 15 hours of the mother's life reached 40.31 B.U. per pituitary or 0.909 B.U. per mg pituitary tissue as compared to the group where the young had been removed for 12 hours and allowed 3 hours' nursing with a content of 32.19 B.U. per pituitary or 0.655 B.U. per mg pituitary tissue. Thus the difference between these 2 groups is 20.2% based on the B.U. per pituitary or 27.9% based on the B.U. per mg pituitary.

The AP of the rabbits after 10 days of lactation also has a high lactogen content (40.00 B.U. per pituitary) while the last 2 groups, after 20 days and 30 days of lactation, respectively, show a gradual decrease. There was a rather conspicuous weight increase of the pituitary after parturition; the pituitary of the non-lactating female rabbits weighed about 35 mg while the 10-day postpartum, lactating rabbits had pituitaries averaging 78.88 mg or more than twice the weight of the non-lactating rabbits.

The lactogen content of the AP of pregnant guinea pigs remained on a level with that of the non-pregnant females; not even in the group just before parturition was a significant rise noticed (Table II). Forty-eight hours after parturition an increase from about 10 to 18.38 B.U. per pituitary was found in the group where the young were removed for 15 hours; the corresponding group in which nursing was permitted the last 3 hours contained 13.13 B.U. per pituitary. Thus in the guinea pig a considerable difference between these two groups was observed. The 10-day lactation group contained 15.19 B.U. per pituitary, the 20-day postpartum group 12.45 B.U., and the 30-day postpartum group 9.63 B.U. per pituitary.

It will be seen that the rise in the content of the lactogenic hormone after parturition is far more conspicuous in the rabbit than in the guinea pig. After the maximum was reached there was a gradual decline with the advance of the lactation period in both species.

Summary. In the case of both the rabbit and the guinea pig the lactogen content of the AP does not increase during early pregnancy and very little during late pregnancy. After parturition a distinct increase is noted in both species although the rise in the case of the rabbit is much higher than in the guinea pig. On the other hand, the increase seems to be somewhat slower and more gradual in nature in the rabbit than in the guinea pig which seems to reach its peak almost immediately after parturition.

In both species there is evidence that the absence of nursing results in an increased lactogen content of the AP whereas following nursing the pituitary contains less lactogen. It has been suggested previously that the stimulus of nursing in some way causes a discharge of lactogen from the pituitary.[1]

10947

Effect of Testosterone Propionate on Genital Tract of Adrenalectomized and Ovariectomized Immature Female Rats.

IRA T. NATHANSON AND RULON W. RAWSON. (Introduced by J. C. Aub.)

From the Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University.

In an earlier communication the effect of testosterone upon the genital tract of ovariectomized and hypophysectomized immature female rats was reported.[1] The essential findings were (a) a direct effect upon the vagina, producing premature opening and an estrous response; (b) a direct effect upon the uterus; and (c) an indirect effect upon the ovary through stimulation of the anterior pituitary. The nature of the vaginal and uterine response to testosterone was not clear, since it has not been shown that this hormone has estrogenic properties. Conversion of testosterone to an estrogen by the adrenal was considered as a possibility. Consequently, in this experiment the effect of testosterone on the genital tract was studied in immature female rats that had been adrenalectomized or both adrenalectomized and ovariectomized. These experiments were performed on immature rats weighing between 55 and 65 g. Maturity generally occurs at a weight of from 110 to 120 g.

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[1] Nathanson, I. T., Franseen, C. C., and Sweeney, A. R., Jr., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 385.

Experiment No. 1. Twelve intact immature female rats were given a single injection of 5.0 mg of testosterone propionate* in sesame oil to serve as controls for the subsequent experiments.

Result: All animals had vaginal opening within 96 hours, uterine enlargement and ovarian stimulation, which was in accord with our previous findings.

Experiment No. 2. Ten immature female rats were bilaterally adrenalectomized and ovariectomized in one operation. They were given a total of 5 cc of 0.9% NaCl subcutaneously in divided doses daily. In addition, 5 of these received 6 dog units of cortin† subcutaneously once within 6 hours after operation. Twenty-four hours following operation 5.0 mg of testosterone propionate were given to each of these 10 animals.

Result: (a) Vaginal opening occurred within 120 hours in all animals. In 7 of these it occurred within 96 hours. Microscopic examination revealed mucification and cornification of the vaginal epithelium. (b) Uterine enlargement and edema was noted in all animals. Microscopic examination showed an increase in the stroma, in vascularity, and in the number of uterine glands.

Treatment with saline was discontinued in all animals immediately after vaginal opening. Weight curves and the survival period were taken as criteria of complete adrenalectomy. All animals except one died within 144 hours after cessation of treatment. The one animal which survived was believed to have sufficient adrenal tissue remaining to maintain life.

Experiment No. 3. Six immature female rats were submitted to bilateral adrenalectomy and were then given 5 cc of 0.9 NaCl subcutaneously in divided doses daily. Six dog units of cortin were given subcutaneously to each animal only once within 6 hours after operation. Twenty-four hours after operation 5.0 mg of testosterone propionate were given subcutaneously.

Results: (a) Vaginal opening occurred in all animals within 96 hours. Microscopic sections showed the changes described above. (b) Uterine changes were also similar. (c) The ovaries showed definite follicle stimulation and in one instance evidence of early corpus luteum formation.

Treatment with saline was discontinued as soon as vaginal opening was noted. All animals died within 72 hours after cessation of this treatment.

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* We are indebted to Doctors Gregory Stragnell and Max Gilbert of the Schering Corporation for a generous supply of testosterone propionate (Oreton).

† Eschatin—Parke, Davis & Co.

Experiment No. 4. Five immature female rats were submitted to bilateral adrenalectomy and were then given 5 cc of 0.9 NaCl subcutaneously in divided doses, daily. No other treatment was given. The animals were sacrificed 7 to 9 days after the operative procedure.

Results: Vaginal opening did not occur in any animal. Histological examination of the vagina, uterus, and ovaries did not reveal any perceptible alteration from the normal untreated controls.

It appears from the results summarized above that testosterone propionate will act directly upon the vagina and uterus of the adrenalectomized, and adrenalectomized and ovariectomized immature female rats. It is also capable of stimulating the ovary by way of the hypophysis[1] even in the absence of the adrenals. The cortin given only once post-operatively may have aided this reaction, but it does not seem probable, since the reaction occurred in 5 rats which had not been so treated. One cannot be absolutely certain that all adrenal tissue was removed, even though a careful autopsy was performed on all the animals. At any rate, they all showed definite adrenal insufficiency with the exception of the one animal. Death occurred invariably during a convulsion. It does not seem probable, therefore, that sufficient adrenal tissue remained to change the chemical structure of testosterone if it could not maintain life.

In general, the animals which had bilateral adrenalectomy and ovariectomy survived longer than those which had bilateral adrenalectomy only.

Conclusions. Testosterone propionate acts directly upon the uterus and vagina and indirectly upon the ovary *via* the hypophysis in operatively adrenal-insufficient immature female rats.‡

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‡ We are indebted to Dr. Joseph C. Aub for valuable suggestions in carrying out this experiment.

10948

No Platelet-Destroying Action in Extracts of the Spleen and Urine of Patients with Chronic Thrombopenic Purpura.*

L. M. Tocantins.

From the Department of Medicine, Jefferson Medical College, Philadelphia, Pa.

The spleen of patients with chronic or "essential" thrombopenic purpura is said to contain a substance which destroys blood platelets. By acetone treatment of such spleens, Troland and Lee[1] have obtained an extract which, when injected into the circulation of rabbits, produced a thrombopenia. Extraction by the same method of other organs, or of spleens of patients with diseases other than thrombopenic purpura, yielded negative results.

The spleens of 6 patients under our observation were extracted by the method as outlined by Troland and Lee. Three of these patients had chronic thrombopenic purpura (with characteristic findings in the sternal marrow), one had portal cirrhosis with congestive splenomegaly, and 2 had the splenohepatic syndrome (Banti's Disease) in an advanced form. The spleens were ground immediately after removal from the body, and the grindings were placed in a volume of reagent acetone equivalent to 3 times the weight of the spleen. Extraction was allowed to proceed for at least 2 months. At the end of this time, the acetone was distilled off by heat and suction, and the residue suspended in distilled water. In 2 instances, the acetone extract was divided into 2 portions; in the first portion the acetone was driven off by heat, in the second portion it was removed by vacuum distillation alone.

In one instance, the oily residue after evaporation of the acetone was extracted with ether and the material remaining after evaporation of the ether suspended in distilled water. An amount of water equivalent in volume to about one-half the weight of the extracted organ was used to take up the residue. In addition to splenic extracts, the urine of patients in the acute stage of chronic thrombopenic purpura was injected intravenously into rabbits, either as whole unmodified urine in 10 cc amounts or as chloroform extracts of the entire 24-hour output. Such extracts were prepared by acidifying the urine with 1% hydrochloric acid to pH 3.0, using Congo Red as indicator and extracting with an equal volume of chloroform in a reflux condenser for about 10 hours. The chloroform was then

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* Aided by a grant from the J. Ewing Mears Research Fund.

[1] Troland, C. E., and Lee, F. C., *J. A. M. A.*, 1938, **111**, 221.

evaporated off and the residue suspended in water or cotton seed oil. The cotton seed oil suspension was injected subcutaneously. Twenty-four-hour specimens of urine after evaporation to dryness were also extracted with acetone or ether or 95% alcohol, and after the solvent was driven off, each of the respective residues was suspended in water and injected intravenously. Chinchilla rabbits of about the same age and weight were used in all experiments. Duplicate platelet,[2] red and white cell counts were performed at various intervals, 6 times on the first day of an injection, twice or more times on the second day, and daily thereafter. A few animals received one single injection of the extract, others received as many as 6 injections of 10 cc each, one each day consecutively.

Results. There were no significant changes in the number of platelets following the injection of the extracts of any of the spleens. A slight leukocytosis, lasting approximately 12 hours was the only change. The injection of ether, acetone or chloroform extracts of the urine was followed likewise by no significant fluctuation in the number of platelets. The alcohol extracts of the evaporated urine when injected in saline suspension, produced necrosis of the tissue around the injection site, convulsions and occasionally death. In none of the surviving rabbits were any significant changes in the number of platelets detected.

Summary. Extraction by various methods of the spleen and urine of patients with "essential" thrombopenic purpura, and injection of these extracts into rabbits, failed to disclose the presence of platelet-destroying substances in the spleen and urine of these patients.

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[2] Tocantins, L. M., *Arch. Path.*, 1937, **23**, 850.

10949 P

Changes in the Anterior Pituitary Gland of Rats With Experimental Goiter. II.

JOSEF WARKANY AND ROSE COHEN NELSON. (Introduced by Glenn E. Cullen.)

From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati.

In a previous communication[1] cellular changes in the anterior lobe of the pituitary gland of rats reared on a goitrogenic diet[2] were described. In male rats fed for 3 to 12 months on the Steenbock and Black diet[3] supplemented by viosterol, nests of large cells were seen in the anterior lobe of pituitary glands fixed in Bouin solution and stained with hematoxylin-eosin. We report now the results of histologic examinations of pituitaries which had been fixed in Regaud

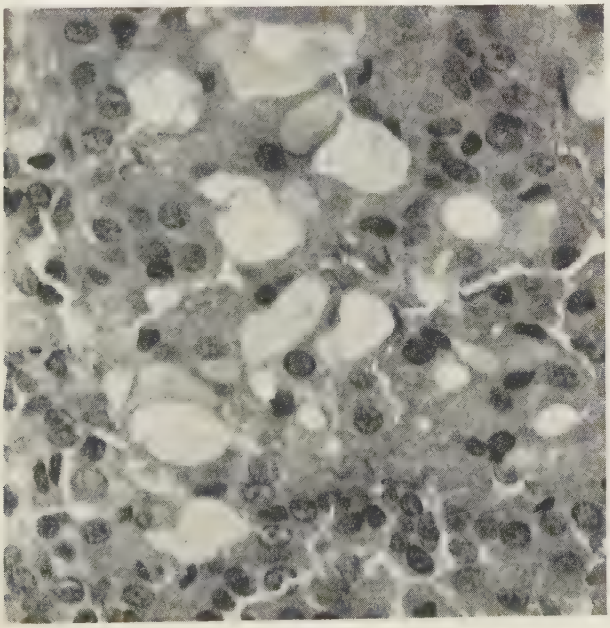

FIG. 1.

Section of anterior hypophysis of rat 130 Y (10.5 months on goitrogenic diet), showing a nest of large cells containing vacuoles. (Regaud fixation; Hematoxylin-Eosin stain; × 800.)

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[1] Nelson, R. C., and Warkany, J., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 66.

[2] Krauss, W. E., and Monroe, C. F., *J. Biol. Chem.*, 1930, **89**, 581; Thompson, J., *J. Nutrition*, 1932, **5**, 359.

[3] Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, **64**, 263.

solution and were stained with methods which permit the identification of granules as well as that of other elements contained in the cytoplasm of the abnormal cells. During life the rats had been treated for 4 to 12 months in the same way and fed the same diet as the animals described before.

On sections of pituitaries fixed with Regaud solution and stained with hematoxylin and eosin, groups of large cells can be recognized with the low power objective of the microscope; the picture resembles that of pituitaries fixed with Bouin solution. With the high power magnification the large cells appear to contain vacuoles of different sizes. In animals on the diet 4 to 6 months numerous small vacuoles can be seen which give the cytoplasm a spongy appearance. In older animals the large cells often contain but a single large vacuole which is filled with a pale hyaline or colloid material (Fig. 1). Mitotic figures are occasionally seen in these cells (Fig. 2) suggesting a process of cellular hyperplasia.

In order to identify the cell granules similar sections were stained by the Cleveland and Wolfe method.[4] The sections of the pituitary of a male rat (137 B) reared on the diet for 9 months, whose weight was 284 g and whose hypertrophic and hyperplastic thyroid weighed

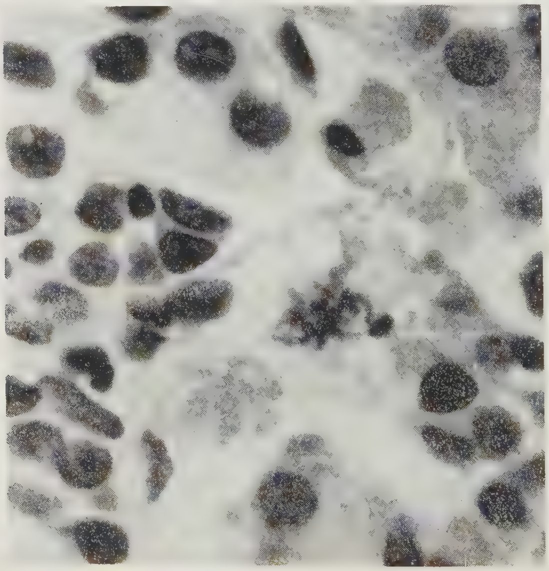

Fig. 2.

Section of anterior hypophysis of rat 129 Y (10.5 months on goitrogenic diet), showing large vacuolated cells, one of which is undergoing mitosis. (Regaud fixation; Hematoxylin-Eosin stain; about × 1800.)

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[4] Cleveland, R., and Wolfe, J. M., *Anat. Rec.*, 1932, **51**, 409.

154 mg when sacrificed, revealed the following findings which are typical: The large cells could be recognized as basophilic cells, some densely packed with blue granules and others containing, in addition, vacuoles of different sizes (Fig. 3). The vacuoles were filled with hyaline material in varying amounts. The larger hyaline areas usually stained light blue; small vacuoles often took a dark blue stain. However, there were numerous large cells in the same part of the section containing hyaline which had taken a red stain. Orange staining hyaline material could be seen in intercellular spaces as well as in the residual cleft. The sections also contained areas of normal appearance made up of cells of normal size and normal granular structure. It should be emphasized that numerous eosinophilic cells can be seen in such areas.

The weight of the testes of the animal whose pituitary is reproduced in Fig. 1 was approximately normal and the epithelium of the prostate was tall, columnar in type and the secretory vacuoles could be plainly seen. This animal successfully fathered 2 litters of living young; the second breeding taking place shortly before the animal was sacrificed.

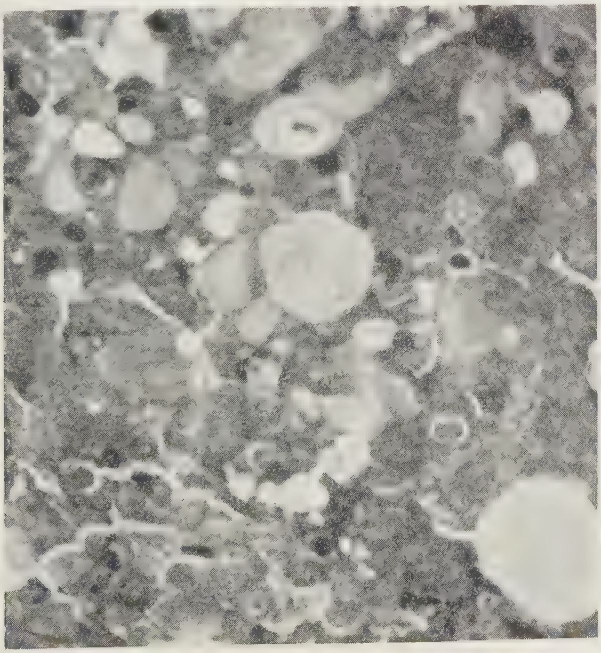

FIG. 3.

Section of anterior hypophysis of rat 137 B (9 months on goitrogenic diet), showing large vacuolated cells and areas of hyaline material. (Regaud fixation; Cleveland and Wolfe stain; × 800.)

As a rule, however, the reproductive organs of animals reared on this diet are slightly retarded in development[1] and breeding occurs infrequently. One could suspect, therefore, that the pituitary changes described by us are secondary to the impaired sex function frequently observed in these animals. Furthermore, the growth of these animals is retarded and one might consider a possible relationship between the impairment of growth and the changed pituitary morphology.[5]

In order to rule out the reproductive irregularities and the retardation of growth as factors in our experiments, we have made use of an improved goitrogenic diet devised by Remington.[6] This diet results in normal, or nearly normal, growth, maturity and reproduction of the experimental animals. The thyroids of these rats, however, resemble in every respect those found in animals fed the supplemented Steenbock and Black diet, which was used in our previous experiments.

Remington's improved goitrogenic diet consists of wheat gluten 18, yellow corn meal 78, calcium carbonate 1, sodium chloride 1, dried pig liver 2. (We added 60 U.S.P. units of vitamin D as viosterol every 10 days.)

The results of Remington were corroborated and we were thus enabled to examine the pituitary glands of goitrous male rats which were satisfactory with regard to growth and reproductive function.

The histological picture of these anterior pituitaries is, as far as we can determine, identical with that seen in animals on the Steenbock and Black diet supplemented with viosterol. Marked pituitary changes were found in 10 male rats fed Remington's improved goitrogenic diet for 27-54 weeks. In pituitaries of 5 animals fed the diet for not more than 14-18 weeks the changes were present in a moderate degree only. This was also true for one rat which was on the diet for 35 weeks. The morphology and the microscopic picture of the testes and prostatic glands of these animals appear to be normal.

In summary it can be stated that in male rats reared on goitrogenic diets, nests of large vacuolated cells filled with a hyaline material are seen in the anterior pituitaries. Mitotic figures are not infrequently found in such cells. These changes seem to be causally related to the changes of the thyroid gland[7] and not to impairment of growth and reproduction.

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[5] Erdheim, J., *Frankfurt. Z. f. Path.*, 1936, **49**, 452; Zeckwer, I. T., *Arch. Path.*, 1938, **25**, 802.

[6] Remington, R. E., *J. Nutrition*, 1937, **13**, 223.

[7] For review of pituitary following thyroidectomy see: Altschule, M. D., and Cooper, P., *Arch. Path.*, 1937, **24**, 443; Zeckwer, I. T., Davison, L. W., Keller, T. B., and Livingood, C. S., *Am. J. Med. Sc.*, 1935, **190**, 145; Severinghaus, A. E., *Sex and Internal Secretions*, p. 1045, Williams & Wilkins, 1939.

10950 P

The Relation of Thyroid to Creatine and Creatinine Excretion in the Rat.*

J. B. Allison, C. Glaser and S. L. Leonard.

From the Bureau of Biological Research, Rutgers University, New Brunswick, N. J.

It is generally stated that creatinuria accompanies hyperthyroidism in mammals and that there is a decrease in the excretion of creatine with hypothyroidism if the mammal excretes creatine normally.[1, 2, 3] Experimental studies on the creatine-creatinine excretion have been made on normal and hyperthyroid rats but none to our knowledge have been made on the thyroidectomized rat. This preliminary note on the creatine-creatinine excretion in normal and thyroidectomized rats is part of a comprehensive study being undertaken on the relationship of the endocrines to creatine-creatinine metabolism.

Methods. Adult male rats weighing about 250-350 g, of the Sprague-Dawley strain, were used and were fed a diet of Purina chow *ad lib.* They were kept in individual metabolism cages and 24-hour samples of urine were collected under toluol. The modified Folin[4] methods for determining creatine and creatinine were used. The amounts of creatine and creatinine excreted during 24-hour periods were expressed in millimoles per kilo of rat.

Experimental. (1) Determinations of creatine and creatinine were made on 5 24-hour urine samples from each of 5 thyroidectomized and 4 control rats over each of 2 2-week periods. The normal level of excretion of these substances was determined from 8 samples for the previous 2 weeks. The control group was subjected to a mock operation at the time the others were thyroidectomized. A summary of the data, presented in Table I, demonstrates that there is a reduction in the excretion of creatine in thyroidectomized rats which is not shown by the controls and that there is more of a tendency for the excretion of creatinine to increase in the thyroidectomized animals than in those subjected to a mock operation.† It is

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* Aided by a grant from the Penrose Fund of the American Philosophical Society.

[1] Kendall, E. C., *Thyroxine*, 1929, Chem. Catalogue Co.

[2] Hunter, A., *Creatine and Creatinine*, 1928, Longmans, Green and Company.

[3] Pugsley, L., Anderson, E., and Collip, J. B., *Biochem. J.*, 1934, **28**, 1135.

[4] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

† The significance of these findings is increased by recent data obtained by Mr. Glaser. He injected 10-20 mg of creatine into 2 thyroidectomized and 2 control rats and recovered approximately 40% less of this excess creatine from the urine of the thyroidectomized than from the control animals. The injection experiments were repeated using creatinine but the excretion of an excess of this substance was unaffected by thyroidectomy.

TABLE I.

Millimoles per kg of Creatine and Creatinine Excreted in 24 Hours by White Rats During a 2-week Period Before, and During a 4-week Period After Thyroidectomy or After Mock Operation Without Thyroidectomy.

Rat No.	Before operation 2 weeks	After thyroidectomy	
	Avg 8 samples creatine millimoles	First 2 weeks Avg 5 samples creatine millimoles	Second 2 weeks Avg 5 samples creatine millimoles
1	.122	.050	—
2	.116	.052	—
3	.082	.054	.048
4	.089	.040	.045
5	.092	.061	.064
Avg	.100	.051	.052
		After mock operation	
6	.088	.076	.070
7	.091	—	.142
8	.118	.105	.130
9	.163	.138	.162
Avg	.113	.106	.126
		After thyroidectomy	
	Avg 8 samples creatinine millimoles	Avg 5 samples creatinine millimoles	Avg 5 samples creatinine millimoles
1	.232	.281	—
2	.180	.226	—
3	.255	.255	.296
4	.254	.255	.263
5	.257	.243	.273
Avg	.236	.252	.277
		After mock operation	
6	.268	.258	.246
7	.238	—	.275
8	.246	.258	.244
9	.222	.235	.240
Avg	.243	.250	.251

possible that the decrease in the excretion of creatine by the thyroidectomized animals is due at least in part, therefore, to a reversible creatine-creatinine reaction.

(2) The 5 thyroidectomized rats were injected with 0.25 mg of thyroxin for 4 days. The averaged data obtained on 3 of these rats studied simultaneously are illustrated in Fig. 1. These data show the effect thyroxin had on all 5 rats. In every case there was a marked increase in the excretion of creatine accompanied by a loss of body weight. The slight decrease in the excretion of creatinine observed in the averaged data 2 to 5 days after the last injection of thyroxin also may be significant.

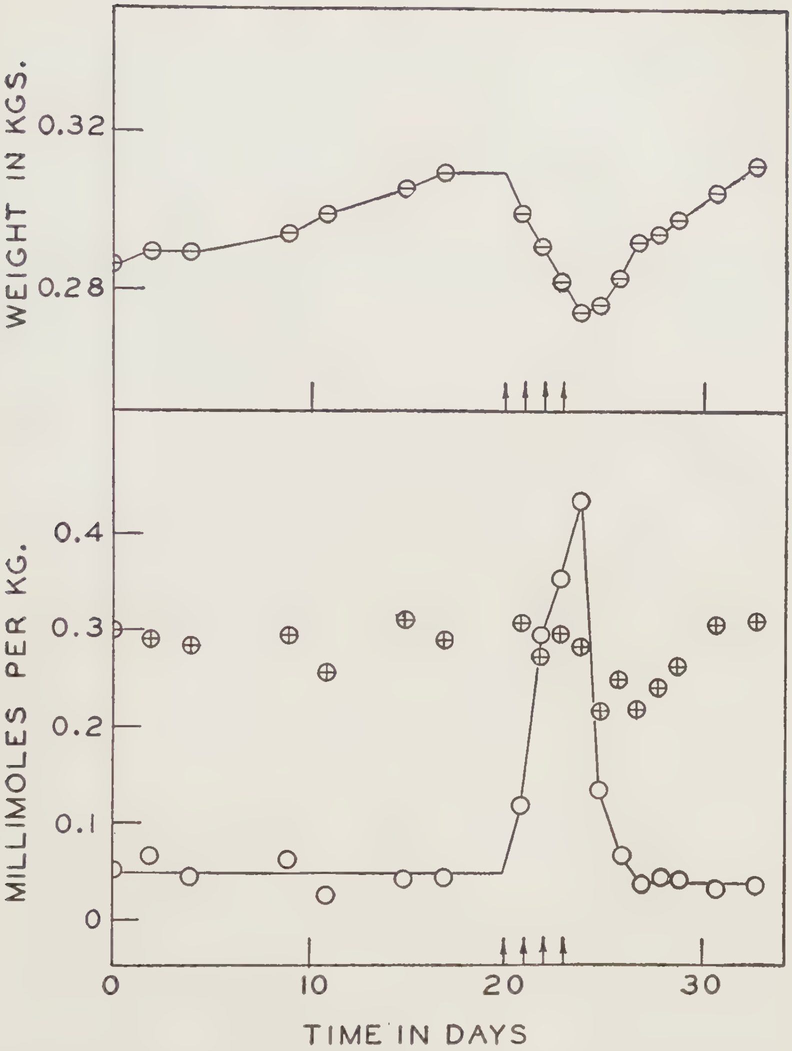

Fig. 1.

The average weights in kilograms ⊖ of 3 thyroidectomized rats are plotted against time in days. The average millimoles of creatinine ⊕ and of creatine ○ excreted per kilogram of body weight in 24 hours are plotted against time in days. 0.25 mg of thyroxin were injected in each rat each day for 4 days, indicated by the arrows.

Summary. Preliminary data demonstrate that (1) thyroidectomy decreases creatine excretion and suggests that this decrease always may be accompanied or followed by an increase in creatinine; (2) injection of thyroxin into thyroidectomized rats increases the excretion of creatine with loss in body weight followed by a slight and probably significant decrease in the excretion of creatinine.

10951 P

Blood Histamine in the Rabbit During Anaphylactic Shock.

Bram Rose* and Paul Weil.* (Introduced by J. S. L. Browne.)
From the McGill University Clinic, Royal Victoria Hospital, Montreal, Canada.

It has been shown by Code[1] that there is an increase in the whole blood histamine of the dog and guinea pig during the stage of acute symptoms in anaphylactic shock. In the dog, this increase was shown to occur mostly in the plasma. Plasma histamine was not determined in the guinea pig. Code and Hester[2] have demonstrated a diminution of the whole blood histamine in the horse and calf during anaphylactic shock.

During the course of investigations on anaphylactic shock in the rabbit, blood histamine determinations were made on the whole blood, and on the plasma.

Two groups of 4 rabbits each were sensitized by the administration of 3 successive doses of 2 cc of horse serum intraperitoneally at 4-day intervals. On the day of the experiment, 3 to 4 weeks after the last sensitizing dose, control specimens of blood were taken from all the animals. In the first group, both whole blood and plasma histamine were determined. In the second group, whole blood histamine alone was estimated.

The shock dose consisted in each case of 6 cc of horse serum given intravenously. Four of the animals were given a dose of cortin one-half hour before the shock dose was administered.

Following the administration of the shock dose of horse serum, a second specimen of blood was removed by cardiac puncture at a time interval varying from 4 to 15 minutes.

The blood samples were obtained by cardiac puncture, and clotting was prevented by the use of heparin (Connaught Laboratories) in all specimens. Blood histamine was determined by the Code[3] modification of the Barsoum and Gaddum method. All assays were carried out on the isolated guinea pig ileum suspended in Tyrode solution containing atropine sulphate in a concentration of 1×10^{-6}. Histamine values are given in terms of the base.

In all cases a marked decrease in the whole blood histamine as

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* Aided by a grant from the Banting Research Foundation.

[1] Code, C. F., *Proceedings Am. Phys. Soc.*, 1938, p. 40.

[2] Code, C. F., and Hester, H. R., *Am. J. Phys.*, 1939, **127**, 71.

[3] Code, C. F., *J. Phys.*, 1937, **89**, 257.

TABLE I.
Histamine Content of Whole Blood and Plasma of Sensitized Rabbit Before and During Anaphylactic Shock.

	γ Histamine/cc Blood		γ Histamine/cc Plasma		
Rabbit No.	Control specimen	Specimen after inj. of antigen	Control specimen	Specimen after inj. of antigen	Reaction
20	2.5	0.3	0.1	0.3	Mild
21	3.0	0.75	0.35	0.125	’’
22*	3.0	1.1	0.175	0.4	Severe, died
23*	1.35	0.5	1.0	0.4	’’ ’’
24*	5.0	0.7			’’ ’’
25	2.75	1.0			’’
26	2.0	0.4			Mild
27*	3.0	0.6			Severe, died

* Animals given cortin before receiving antigen.

TABLE II.
Histamine Content of Whole Blood and Plasma of Normal Rabbit.

	γ Histamine/cc	
Rabbit No.	White Blood	Plasma
2	2.5	0.5
3	2.75	0.3
4	2.0	0.5
6	2.75	1.0
7	2.5	0.45
8	2.5	0.75
9	3.5	7.0
10	2.5	1.0

compared with control values was observed. (Table I.) In the 4 animals in which plasma histamine was determined along with the whole blood histamine, minor changes were noted but these were not consistent and did not differ markedly from the control values or from plasma histamine found under normal conditions. (Table II.)

A curious phenomenon was observed in that the animals which received cortin all died within 7 minutes of the injection of the shock dose in acute anaphylactic shock. The other animals exhibited mild to severe symptoms, but did not die.

The results obtained in this experiment differ from those reported by Code[1] in which an increase of the total blood and plasma histamine was observed. They are, however, similar to the changes occurring in the total blood histamine during anaphylactic shock in the horse and calf, which Code and Hester[2] have recently reported.

It is difficult to interpret such results at the moment. Code[4] has shown that the white blood cell layer of the blood of the rabbit contains up to 80% of the total blood histamine, and this has been confirmed by one of us.[5] It may be that during anaphylactic shock, such histamine is liberated into the plasma where it is free to exert its effect, and from which it is rapidly removed by various tissues, such as occurs in the rat (Rose and Browne[6]) or that the rapid disappearance is coincident with the sudden withdrawal of white blood cells from the circulation during anaphylactic shock in the rabbit. It should be noted that there is no correlation between the severity of the symptoms and degree of change in the histamine content of the blood as observed in these preliminary experiments. The relationship of the cortin to anaphylaxis will be discussed elsewhere.

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[4] Code, C. F., *J. Phys.*, 1937, **90**, 485.

Summary. During acute anaphylactic shock in the rabbit, a marked decrease in total blood histamine occurs. No increase of plasma histamine is observed.

10952

Effect of Cyanide on Cerebral Metabolism.*

J. F. FAZEKAS, HARRIET COLYER AND H. E. HIMWICH.

From the Department of Physiology and Pharmacology, Albany Medical College, Union University, Albany, New York.

Loevenhart[1] suggested the use of small doses of cyanide intravenously in the treatment of schizophrenia. Since many methods now employed for treating schizophrenia (metrazol,[2] insulin,[3] and nitrogen[4]) depress cerebral metabolism the present study was made to determine the effect of small concentrations of cyanide on cerebral metabolism *in vivo*. In addition a comparison was made between the effects of intravenous cyanide on cerebral and muscular metabolism.

Method. Dogs were anesthetized with pentobarbital and the superior longitudinal sinus, femoral vein and artery were exposed. Blood was drawn before and for various periods after the intravenous injection of potassium cyanide. The blood samples were then analyzed for oxygen.[5] The difference in oxygen contents of the femoral artery and superior longitudinal sinus represent the cerebral oxygen consumption, chiefly of the cerebral cortex; the differences between femoral artery and vein and the oxygen uptake of muscle.

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[5] Rose, B., unpublished results.

[6] Rose, B., and Browne, J. S. L., *Am. J. Phys.*, 1938, **124**, 412.

* Aided by a grant from the Child Neurology Research (Friedsam Foundation).

[1] Loevenhart, A. S., Lorenz, W. F., Martin, H. G., and Malone, J. F., *Arch. Int. Med.*, 1918, **21**, 109.

[2] von Meduna, L., *Z. f. g. ges. Neur. u. Psychiat.*, 1935, **152**, 235.

[3] Sakel, M., *Nerv. and Ment. Dis.*, Monograph No. 62, New York, 1937.

[4] Himwich, H. E., Alexander, F. A. D., and Lipetz, Basile, PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 367.

Results. Thirty-seven observations were made on 11 dogs and typical results are presented. Table I reveals the effects on cerebral metabolism of a large dose of potassium cyanide injected into the common carotid artery. A severe and prolonged depression of oxygen uptake is observed and is still greatly reduced 1½ hours after the injection. In Table II are seen the effects of a small injection followed by a larger dose of potassium cyanide, both injected into the femoral vein. The larger amount produced a more profound depression of oxygen utilization. A dose of cyanide which inhibits

TABLE I.
Effect of Potassium Cyanide on Cerebral Respiration.

Vol. % of Oxygen				
Arterial	Venous	Differences	Time and Amount	Remarks
15.93	7.02	8.91	3 min before KCN	KCN injected into carotid artery
16.92	16.34	.58	2 min after 20 mg KCN	
16.37	15.67	.70	18 min after injection	
15.67	10.96	4.71	1 hr 32 min after injection	

TABLE II.
Effect of Potassium Cyanide on Oxygen Uptake of Brain.

Vol. % of Oxygen				
Arterial	Venous	Differences	Time and Amount	Remarks
14.38	9.12	5.26	Before KCN	KCN injected into femoral vein
12.09	9.85	2.24	7 min after 5 mg KCN	
14.86	8.11	6.75	1 hr after KCN	
15.89	14.56	1.33	10 min after 15 mg KCN	

TABLE III.
Effect of Potassium Cyanide on Brain and Muscle.

Vol. % of Oxygen				
Arterial	Venous	Differences	Time and Amount	Remarks
15.20	B 9.69	B 5.51	7 min before KCN	KCN injected into femoral vein
	M 5.95	M 9.25		
16.83	B 14.33	B 2.50	After 5 mg KCN	
	M 6.15	M 9.68		
15.72	B 11.68	B 4.04	7 min after injection KCN	
	M 4.94	M 10.68		

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[5] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

markedly brain metabolism is without apparent effect on muscle (Table III).

From the results it may be seen that cyanide in various concentrations inhibits cerebral metabolism *in vivo*. If a suitable concentration is administered a depression equal to or greater than that resulting from either metrazol or insulin or nitrogen may be obtained. The underlying mechanism with each agent, however, is different. With metrazol the cause of the cerebral metabolism may be attributed to an arrest of respiratory movements due to severe convulsions. Blood, therefore, passes through the lungs in an unoxygenated form and the brain is deprived of its oxygen supply. In the case of insulin hypoglycemia the diminished concentration of sugar, the chief substrate of the brain, is the underlying factor for the depression of cerebral metabolism. In the nitrogen therapy the decreased cerebral oxygen uptake is due to the replacement of oxygen by nitrogen in the inspired air. With cyanide, on the other hand, a direct poisoning of the enzyme oxidase prevents the combination of the substrate with oxygen. Thus, we see that all treatments thus far used for schizophrenia diminish in one manner or another cerebral oxidations.

Since a large concentration of cyanide causes a marked inhibition of cerebral respiration *in vivo,* it may be concluded that a cyanide-sensitive system may account for the greatest part of cerebral respiration.

It is surprising to note that concentrations of cyanide which inhibit cerebral respiration nevertheless exert no significant decrease on the oxygen consumption of muscle. Several explanations may be ventured. It is possible that qualitatively a smaller reserve of the cyanide-sensitive oxidase enzyme is available in the brain. Thus for the same concentration of cyanide a greater proportion remains in active form in muscle. It is not likely that the cyanide fails to penetrate muscle nor that changes of blood flow different in muscle and brain can explain the differential effect on these two tissues.

Summary and Conclusions. The effects of intravenous and intracarotid cyanide injection on cerebral and muscle metabolism were studied; 37 observations were made on 11 dogs. A significant decrease in the cerebral oxygen consumption was observed, a decrease which varied directly with the concentration of cyanide used. The concentration of cyanide which depressed metabolism of brain failed to exert an effect on the arterial-venous difference of muscle blood. The significance of these findings is discussed.

10953

Preservation of Viable Malaria Parasites in the Frozen State.

L. T. COGGESHALL.

From the Laboratories of the International Health Division of the Rockefeller Foundation, New York.

The study of malaria has often been seriously handicapped by the lack of a suitable method for the maintenance of parasites in a viable state outside a living host. All attempts to cultivate them *in vitro* have been unsatisfactory, as have various freezing and drying procedures which generally give satisfactory results with viruses and bacteria. An important contribution to the problem of preservation of treponematas was made by Turner[1] when he showed that syphilis spirochetes can be kept virulent and active for more than a year in glass containers immersed in a mixture of alcohol and CO_2. Numerous attempts were made, with negative results, in this laboratory to preserve malaria parasites in a viable state by a similar procedure. In these attempts infected blood and tissues from avian, monkey and human sources, as well as infected mosquitoes, were used, and the preserved material, although frozen rapidly, was thawed slowly for the infectivity test. Recently Turner,[2] after his experience with the preservation of treponematas, emphasized the importance of freezing and thawing the material as rapidly as possible and the necessity for low storage temperatures within a very narrow range of fluctuation.

With the thermos jugs used in this laboratory for storage of biologically active material in a mixture of alcohol and solid CO_2, a fairly uniform temperature can be maintained only when there is an excess of CO_2; but as the amount of CO_2 diminishes through evaporation, the temperature of the mixture rises, and under ordinary storage conditions wide variations in the temperature occur. To overcome this difficulty Horsfall[3] recently has devised a dry low temperature storage cabinet in which the total fluctuation in the temperature does not exceed 8°C from the average of −76°C. In investigating once more the possibility of preserving malaria parasites in frozen state, use was made of this cabinet for storage purpose, and the practice of freezing and thawing the material as rapidly as possible was routinely adopted. The results obtained form the basis of this report.

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[1] Turner, T. B., *J. Exp. Med.*, 1938, **67**, 61.

[2] Turner, T. B., personal communication.

[3] Horsfall, F. L., Jr., in press.

Materials. The material for freezing was infected whole blood from rhesus monkeys with subacute or chronic malaria infections. The plasmodia used were *Plasmodium knowlesi* and *Plasmodium inui.* The blood to be frozen was either defibrinated, heparinized, or citrated, using one part of a 2% sodium citrate solution to 3 parts of blood.

Freezing and Thawing. The blood was placed in screw-capped celluloid tubes 4⅞ inches in length and ⅞ of an inch in diameter, with a capacity of 17 cc. In order to freeze the blood as rapidly as possible, the amounts were limited usually to 1 cc. With a whirling motion each tube was immersed in a mixture of solid CO_2 and alcohol. As soon as the material was frozen solid it formed a coating on the inside of the tube. It was then rapidly transferred to a low temperature storage cabinet until ready for testing. The cabinet mentioned above kept cold by dry solid CO_2, maintained a temperature between –72°C and –80°C. Considerable precaution was exercised in order to thaw the frozen specimens as rapidly as possible. Immediately upon removal from the cabinet, the tubes were immersed and rapidly rotated in a water bath at 37.5°C placed beside the cabinet. In this way the contents were reduced to a liquid state at body temperature within a few seconds. Before being injected interperitoneally into normal monkeys, samples were examined for dissolved hemoglobin, number of intact red cells, and parasites.

Determination of Viability of Frozen Parasites. The details of the tests for the viability of the malaria parasites maintained at low temperatures are summarized in Table I. Monkeys 1 and 2 were inoculated with 1 cc of infected *P. knowlesi* and *P. inui* blood which had been stored at –76°C for 70 days, and each animal devel-

TABLE I.
Results of Infectivity Test in Normal Rhesus Monkeys, After Injection with Malaria Parasites Stored at —76°C.

No. of Monkey	Species of parasite	Anti-coagulant used	Conc.[*] of parasites in preserved blood	Days in frozen state	Amt of preserved blood tested, cc	Parasites in blood of monkeys on
1	*P. knowlesi*	Citrate	113	70	4	7th day
2	*P. inui*	’’	+	70	1	14th ’’
3	*P. knowlesi*	Heparin	490	19	1	5th ’’
4	’’ ’’	Defibrinated	490	19	1	5th ’’
5	’’ ’’	Citrate	490	19	1	6th ’’
6	’’ ’’	None[†]	490	19	1	5th ’’
7	’’ ’’	None[‡]	490	19	1	5th ’’
8	’’ ’’	Heparin	+	19	1	9th ’’
9	’’ ’’	Citrate	+	19	1	14th ’’

[*] Number of parasitized red cells per 10,000 normal cells.
[†] Placed in icebox before clotting.
[‡] Allowed to clot before transferring to icebox.

oped typical infection characteristic of the respective organisms. Monkeys 3 through 9 likewise became infected after receiving infected blood from different sources maintained at the same temperature for 19 days. Of particular interest were Monkeys 2, 8, and 9, which became infected from 1 cc of blood in spite of the fact that the source monkeys had a maximum of less than one parasite per 10,000 red cells. The incubation periods observed in all of the monkeys were approximately the same as those obtained from comparable inoculations of fresh blood, thus indicating that there is relatively little loss of infectivity by freezing in the manner described. The data at hand indicate that the method as described for the preservation of malaria parasites of monkeys offers a more practical method than animal passage for maintaining strains or species. The possibilities whereby this method may facilitate the study of malaria are apparent.

Summary. A method of preserving monkey malaria parasites for as long as 70 days by rapid freezing, storage at –76°C, and rapid thawing without loss of viability is described.

10954 P

Relation of Age to Immune Response of Mice to Formolized Equine Encephalomyelitic Virus.

Isabel M. Morgan (Introduced by Peter K. Olitsky.)

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

This paper presents a study of the relation of age to the ability of animals to be immunized to a viral disease, as studied by injecting mice with non-infective formolized equine encephalomyelitic virus.[1] The immunity induced was measured by resistance to active virus and by development of neutralizing antibodies.

The capacity to form antibodies to non-infective antigens of non-viral nature is known to increase with age. This has been demonstrated with phenolized typhoid bacilli in guinea pigs and rats,[2] formolized typhoid bacilli, sheep red cells, horse serum and egg-albumin in rabbits,[3] and formolized *Trypanosoma lewisi* in rats. In the last instance the older rats were shown to have developed also a greater resistance to infection.[4]

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[1] Cox, H. R., and Olitsky, P. K., *J. Exp. Med.*, 1936, **63**, 745; Olitsky, P. K., and Morgan, I. M., *J. Am. Vet. Med. Assn.*, 1939, **95**, 530.

[2] Kligler, I. J., and Olitzki, L., *Z. Hyg. u. Infektionskrankh.*, 1929, **110**, 459.

[3] Freund, J., *J. Immunol.*, 1930, **18**, 315.

Age is known to be a factor in the susceptibility of mice to the virus of equine encephalomyelitis, for when virus is injected intraäbdominally, young mice are highly susceptible, while old ones are almost entirely resistant. Using the intracerebral route, on the other hand, old and young are equally susceptible.[5]

The vaccine used for immunization[1] was a 10% suspension of brain of mice succumbed to infection with the virus of Eastern equine encephalomyelitis (E.E.E.), in 0.5% formalin. Before formolization the titer of the viral suspension was 10^{-9}, as tested intracerebrally in mice; after formolization, no active virus was detectable.

Albino mice of Rockefeller-Institute strain were of 5 age-groups, namely, 2–, 10–, 15–, 30– and 180– days, respectively, at the beginning of immunization. Each group consisted of about 20 mice to be injected, with 12 controls of the same age. The 2-day-old group was started with a larger number, since only a small proportion survive the procedure of immunization. 0.2 cc formolized virus was injected intraäbdominally on the 1st, 3d, and 5th days of immunization. On the 12th day all mice were bled from the tail and the sera of each group pooled. On the 13th day, treated mice of all age-groups were tested for active immunity by injection of tenfold dilutions (4 mice for each dilution) of active E.E.E. virus. Controls were similarly injected to determine the titer of the virus for each age-group. An additional group of mice 2 days old at the beginning of immunization, as well as untreated controls, were injected by the intraäbdominal route. The sera of the immunized mice were tested

TABLE I.
Active Immunity and Serum Neutralizing Antibodies in Mice Immunized with Formolized Equine Encephalomyelitic Virus.

Age of mice at beginning of immunization	No. of intracerebral doses resisted	No. of intraäbdominal doses resisted	No. of doses neutralized by serum
2 days	0	1,000	0
10 ”	10		>100
15 ”	10		10,000
30 ”	100,000		>100,000
6 months	10,000,000		100,000

>100 = at least 100 doses.

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[4] Culbertson, J. T., and Kessler, W. R., *Am. J. Hyg.*, 1939, **29**, 33, Sec. C. For a general review of the subject, see Baumgartner, L., *Yale J. Biol. and Med.*, 1934, **6**, 403.

[5] Sabin, A. B., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 597.

for neutralizing antibodies to E.E.E. virus by the intraäbdominal method in 14-day-old mice,[6] using sera of mice of the same ages as controls. The results are shown in Table I.

Thus, by the intracerebral route, the 2-day-old mice showed no immunity, the 10- and 15-day-old resisted 10 minimal cerebral lethal doses, the 30-day and 6-months, strikingly more, *viz.*, 100,000 and 10,000,000 doses, respectively. However, that the 2-day-old mice were not entirely without response to the formolized virus was shown by the development of resistance to 1,000 minimal lethal intraäbdominal doses. Repetition of these experiments yielded similar results.

Of the sera collected prior to immunity test, that of 2-day-old mice showed no neutralizing antibodies. The capacity to form neutralizing antibodies increased with age, from 10 up to 30 days, when a maximum of 100,000 doses neutralized was reached.

Summary. The ability of mice to be immunized by means of formolized virus of Eastern equine encephalomyelitis increases with age, as shown by the strikingly higher resistance of older immunized mice to the intracerebral injection of active virus, as well as by the amount of neutralizing antibodies developed.

10955 P

Histology of the Cutaneous Reaction in Guinea Pigs to Purified Brucella Protein.

P. MORALES-OTERO, E. KOPPISCH AND L. M. GONZALEZ (Introduced by D. H. Cook.)

From the School of Tropical Medicine, University of Puerto Rico, under the auspices of Columbia University.

Recently, two of us[1] investigated cutaneous hypersensitiveness to Brucella among milkers and cattle handlers using purified Brucella protein (PBP) as antigen. In this paper we describe the histopathology of this test in normal and sensitized guinea pigs.

One group of guinea pigs was sensitized by the intraperitoneal injection, 3 weeks before the test, of 0.2 cc of a suspension of Brucella bacilli obtained by the emulsification of a 48-hour agar culture in 3 cc of normal saline; these animals had all developed cutaneous hypersensitiveness by the time the tests were carried out. Five sensitized and 5 normal guinea pigs were injected intracutaneously in the shaven skin of the abdomen with 3 different dilutions each of PBP prepared as described previously.[2] The dilutions were so made that the injected volume (0.1 ml) of fluid contained 0.1, 0.01 and 0.005 mg of PBP, respectively. As a control, the same animals were injected with a suspension (corresponding to No. 2 McFarland nephelometer) of killed Brucella organisms.

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[6] Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68**, 173.

[1] Morales-Otero, P., and González, L. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 703.

A normal and a sensitized animal were killed at intervals of 6, 12, 24, 48, 72 and 96 hours. The sites of injection were excised so as to include all of the abdominal wall, fixed in Zenker's fluid, embedded in paraffin and stained with hematoxylin and eosin.

The unsensitized animals showed no response grossly; the sensitized ones reacted strongly with erythema and edema at the sites of inoculation, beginning at 6 hours, reaching a maximum at 48 and then regressing slowly.

Histologically, the unsensitized guinea pigs showed, at the site of injection, a slight mainly perivascular infiltration of the derma and subcutaneous fat with eosinophils and neutrophils. The reaction was already at its height 6 or 12 hours after injection and was accompanied by edema with the largest dose only. The outermost intermuscular plane always responded with a leukocytic infiltration predominantly eosinophilic. Beginning at 12 hours, monocytes and lymphocytes replaced the granulocytes, and some fibroblastic proliferation became evident deep in the derma, in the subcutaneous fat and in the outermost intermuscular plane. Qualitatively the histologic response in unsensitized animals was the same with dead bacilli as with PBP. With the latter, the reaction was always strongest with the highest dose, and the results with this dose were quite comparable in kind and degree with those evoked by dead bacilli.

The response provoked by dead bacilli and PBP in sensitized guinea pigs was characterized in all animals and with all doses by a very violent acute inflammatory reaction in the derma and subcutaneous layer at the site of inoculation, extending in some instances to the peritoneum. In the derma and subcutaneous tissue there was, with dead bacilli, very marked infiltration with neutrophils, mainly perivascularly, at 6 hours, but becoming almost massive at 72 hours, which marked the height of the process. Eosinophils were always very scanty, while edema was very marked. Foci of liquefaction of the exudate without frank abscess formation were evident at this time. At 96 hours, although the reaction was still fairly marked, numerous large mononuclear cells and lymphocytes had made their appearance, and the number of neutrophils was much reduced. The response to Brucella proteins was like that to dead bacilli, qualitatively. In degree, the highest dose of 0.1 mg acted very similarly to dead bacilli. With both inocula there was a very sharp drop in the amount of edema between the 72nd and 96th hours. The tissues in the outermost intermuscular plane responded with edema to a similar degree as the corium and subcutaneous layer, but eosinophils always predominated in these tissues, and lymphocytes and monocytes appeared in numbers at least 24 hours before they did in the other layers. Subperitoneal changes were limited to fibroblastic proliferation without leukocytic infiltration.

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[2] Morales-Otero, P., and González, L. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 703.

Summary. Dead Brucella organisms and Brucella proteins injected intradermally in normal guinea pigs provoke an immediate but mild and evanescent inflammatory reaction with eosinophils and neutrophils, and without edema. In sensitized animals the local reaction is more violent, mainly neutrophilic and accompanied by marked edema. It rises to its height the 3rd day after injection and then rapidly regresses. Qualitatively, the changes provoked by Brucella protein are similar to those induced by dead bacilli.

10956 P

Effect of Desoxycorticosterone Acetate* upon Blood Sugar and Electrolytes of Adrenalectomized Rats.

Harold E. Harrison and Helen Coplan Harrison. (Introduced by S. Z. Levine.)

From the New York Hospital and the Department of Pediatrics, Cornell University Medical College.

Following the synthesis of desoxycorticosterone by Steiger and Reichstein,[1] it was shown by Thorn, *et al.*,[2, 3] and Levy-Simpson[4] that this compound is effective in the treatment of adrenal insufficiency, both in adrenalectomized animals and in patients with Addison's disease. The studies thus far reported have shown that the effects of desoxycorticosterone acetate upon the concentrations of serum sodium and potassium, and upon plasma volume are identical with those of adrenal cortical extracts.

Adrenal cortical extracts as well as crystalline compounds isolated from the adrenal cortex have been shown by Long and Katzin[5] to increase the carbohydrate stores of fasted normal and hypophysectomized rats. No data concerning the effect of desoxycorticosterone acetate upon carbohydrate metabolism have as yet been reported.

We have studied the blood sugar, non-protein nitrogen, and serum sodium and potassium of adrenalectomized rats treated with desoxycorticosterone. Blood sugar was determined by the Shaffer, Hartman, Somogyi method,[6] non-protein nitrogen by the Koch and McMeekin method,[6] serum sodium by the Butler and Tuthill method,[6] and serum potassium by the method of Harrison and Darrow.[7]

Male rats of approximately 175 g in weight were adrenalectomized

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* A solution of desoxycorticosterone acetate in sesame oil, supplied through the courtesy of Dr. Erwin Schwenk of the Schering Corporation, was used in these experiments.

[1] Steiger, M., and Reichstein, T., *Nature*, 1937, **139**, 925.

[2] Thorn, G. W., Engel, L. L., and Eisenberg, H., *J. Exp. Med.*, 1938, **68**, 161.

[3] Thorn, G. W., Howard, R. P., Emerson, K., and Firor, W. M., *Bull. Johns Hopkins Hosp.*, 1939, **64**, 339.

[4] Levy-Simpson, S., *Lancet*, 1938, **2**, 557.

[5] Long, C. N. H., and Katzin, B., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 516.

[6] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Vol. II, Methods, 1932, Baltimore, Williams and Wilkins.

[7] Harrison, H. E., and Darrow, D. C., *J. Biol. Chem.*, 1937, **121**, 631.

TABLE I.
Blood Sugar and Serum Electrolytes of Adrenalectomized Rats Fasted 48 Hours.

		mg/100 ml		m.Eq./l	
Treatment	No. of rats	Blood sugar	Blood N.P.N.	Serum Na	Serum K
1. Unoperated controls	6	65 ± 3.3*	44	140.5	5.2
2. Adrenalectomized, untreated	7	48 ± 4.2	70	130.4	6.6
3. Adrenalectomized, sodium salts	7	38 ± 5.0	40	128.8	6.2
4. Adrenalectomized, desoxycorticosterone 1.25 mg daily	6	41 ± 2.6	47	138.0	5.4
5. Adrenalectomized, desoxycorticosterone 2.5 mg daily	9	63 ± 2.9	44	137.5	4.7

* Mean ± standard error of the mean.

The significance of the differences between the mean concentrations of blood sugar were tested using Fisher's[8] Table of t for small samples. The differences between groups 1 and 2, 2 and 5, and 4 and 5 are considered significant.

[8] Fisher, R. A., *Statistical Methods for Research Workers*, 1932, London, Oliver and Boyd.

and maintained for 5 days on a diet of Purina Fox Chow and sodium chloride and sodium bicarbonate in the drinking water. They were then fasted 48 hours and on each of the 2 days injected subcutaneously with desoxycorticosterone acetate in doses of 1.25 mg or 2.5 mg. One control group of adrenalectomized animals was fasted and given no treatment during the experimental period; a second group was fasted and injected intraperitoneally with a solution of sodium chloride and sodium bicarbonate. A group of fasted unoperated controls was also studied. At the end of the 48-hour period of fasting the animals were anesthetized with nembutal and blood was taken from the abdominal aorta for analysis.

From the results given in Table I it is seen that untreated adrenalectomized rats fasted for 48 hours show a definite decrease in concentration of blood sugar, an increased concentration of blood non-protein nitrogen and serum potassium, and a decreased concentration of serum sodium. Treatment with sodium salts does not influence the blood sugar but prevents the rise in non-protein nitrogen. The treatment, however, was not sufficient to maintain normal concentrations of serum sodium. The injection of 1.25 mg of desoxycorticosterone acetate per day has no effect upon the blood sugar of adrenalectomized rats, although this dose is sufficient to maintain normal concentrations of serum electrolytes and non-protein nitrogen. The concentrations of blood sugar in the adrenalectomized rats are maintained at normal levels by the injection of 2.5 mg of desoxycorticosterone daily.

Conclusions. The injection of adequate amounts of desoxycorticosterone acetate into fasted adrenalectomized rats does prevent the drop in concentration of blood sugar found in untreated adrenalectomized rats. The amount required to maintain the blood sugar is greater than that necessary to maintain normal concentrations of serum electrolytes and non-protein nitrogen.

10957

Effect of Local Applications on Development of Ultraviolet Tumors.*

HAROLD P. RUSCH, CARL A. BAUMANN AND BERNERD E. KLINE.
(Introduced by W. J. Meek.)

From the Department of Physiology, University of Wisconsin Medical School, Madison.

We have previously demonstrated that the rate of tumor production by ultraviolet light varies with the time of irradiation, with the amount of pigment in the skin, and with the character of the diet.[1, 2] The present study deals with the effect of various substances on the development of U. V. tumors, when applied directly to tissues in which neoplastic changes are occurring.

Three types of materials were used: oils, oxidizing agents, and carcinogenic compounds. Oils were studied because previous work had shown that the production of U. V. tumors was markedly accelerated by the consumption of a high-fat diet (30% Crisco), on which the fur became definitely greasy.' The acceleration, therefore, could have been due to either a local or a systemic effect. By painting oil on the ears of irradiated mice on a normal diet the local factor alone could be studied.

The oxidizing agents were studied because of Roffo's emphasis of the importance of cholesterol in tumor production. He has shown that irradiated cholesterol fogs a photographic plate[3] and claims that this reaction is analogous to that taking place within the skin, when an animal is exposed to excess amounts of ultraviolet light. Stavely and Bergmann[4] and Mayneord and Roe[5] have demonstrated

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* Supported in part by the Jonathan Bowman Cancer Fund and the Wisconsin Alumni Research Foundation.

[1] Rusch, H. P., and Baumann, C. A., *Am. J. Ca.*, 1939, **35**, 55.

[2] Baumann, C. A., and Rusch, H. P., *Am. J. Ca.*, 1939, **35**, 213

[3] Roffo, A. H., *Am. J. Ca.*, 1933, **17**, 42.

[4] Stavely, H. E., and Bergmann, W., *Am. J. Ca.*, 1937, **30**, 749.

[5] Mayneord, W. V., and Roe, E. M. F., *Am. J. Ca.*, 1937, **31**, 476.

that irradiated cholesterol contains peroxides which are slowly liberated, and that the fogging of a photographic plate by irradiated cholesterol is due to these liberated peroxides. We have, therefore, painted known peroxides, as well as cholesterol solutions, on the ears of mice developing U. V. tumors.

Carcinogenic compounds were studied as a means of obtaining information on the interrelation of cancer-producing agents such as chemicals and light. This interrelationship is as yet poorly understood, and available data are both incomplete and contradictory. Schorr and Ssobolewa[6] and Vles, DeCoulon, and Ugo[7] report a more rapid development of tumors due to tar painting in mice kept in the light as compared to those kept in the dark. Bang,[8] Lipschütz,[9] and Seelig and Cooper,[10] however, failed to find any such difference. Findlay[11] found that cancer development in tarred mice was accelerated by ultraviolet light, and Büngeler reported a similar effect due to sunlight.[12] However, Kohn-Speyer[13] failed to confirm Findlay; and Taussig, Cooper, and Seelig[14] observed that ultraviolet light did not stimulate tumor production in mice painted with benzpyrene. The discrepancies are no doubt partly due to differences in the relative amounts of light and carcinogenic agents employed. In our present study the amount of light used was by itself sufficient to produce tumors in from 4 to 6 months.

Tumors were produced in mice by irradiation with ultraviolet light, and during the induction period, various substances were painted on the ears, *viz.,* those tissues in which the tumors were developing. The technic of irradiation was essentially that described previously.[1] Young adult strain C mice† of both sexes were used throughout. They were placed in groups of 20 to 25 and were irradiated in cylindrical wire mesh cages for 45 minutes daily, at a distance of 50 cm from an air-cooled mercury vapor lamp. The experiments were run in series over a period of 16 months. Each series

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[6] Schorr, G., and Ssobolewa, N., *Z. f. Kreb.*, 1930, **31**, 308.

[7] Vles, F., DeCoulon, A., and Ugo, A., *Compt. rend. Acad. d. Sc.*, 1931, **193**, 893.

[8] Bang, F., *Compt. rend. Soc. de Biol.*, 1922, **87**, 754.

[9] Lipschütz, B., *Arch. Dermat. u. Syph.*, 1924, **147**, 161.

[10] Seelig, M. G., and Cooper, Z. K., *Surg., Gyn. and Ob.*, 1933. **56**, 752.

[11] Findlay, G. M., *Lancet*, 1928, **2**, 1070.

[12] Büngeler, W., *Z. f. Krebs.*, 1937, **46**, 130.

[13] Kohn-Speyer, A. C., *Lancet*, 1929, **2**, 1305.

[14] Taussig, J., Cooper, Z. K., and Seelig, M. G., *Surg., Gyn. and Ob.*, 1938, **66**, 989.

† Obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

TABLE 1.

Effect of Oils, Carcinogens and of Peroxides on Tumor Production.

Substance	Conc. and solvent	No. of mice at start	No. of mice at time of first tumor	Animals rayed	Chemicals rayed	% of animals with tumors									
						3 Mo.	3½ Mo.	4 Mo.	4½ Mo.	5 Mo.	5½ Mo.	6 Mo.	6½ Mo.	7 Mo.	7½ Mo.
Controls	—	65	57	+	—	0	6	17	29	52	63	70	90	—	—
Mineral oil	100%	40	40	+	0	5	22	49	89	98	—	—	—	—	—
,, ,,	100%	25	21	0	+	0	0	0	0	0	0	0	0	0	0
Cholesterol	3% in cottonseed oil	20	19	+	0	0	28	44	72	100	—	—	—	—	—
Cottonseed oil	100%	40	38	+	0	0	5	22	56	77	85	—	—	—	—
Olive oil	100%	20	18	+	0	0	6	28	56	83	—	—	—	—	—
Wheat germ oil	Crude ether extr.	20	19	+	0	0	11	17	50	83	—	—	—	—	—
Linseed oil	100%	20	20	+	0	0	5	10	20	40	—	—	—	—	—
Methylcholanthrene	0.2% in benzene	20	20	0	0	0	0	10	30	50	60	70	100	—	—
,,	0.2% in cottonseed oil	20	17	0	0	0	6	12	18	47	56	65	71	—	—
,,	0.2% in mineral oil	20	19	0	0	0	0	0	0	33	48	55	78	—	—
1,2,5,6 Dibenzanthracene	0.3% in benzene	21	21	+	0	0	5	15	39	50	66	66	83	—	—
,, ,,	,, ,, ,,	21	19	0	0	0	0	0	0	0	0	0	12	—	—
,, ,,	,, ,, ,,	21	20	0	+	0	0	0	0	0	0	0	0	0	0
Controls*	—	25	24	+	0	0	4	8	10	10	30	45	50	60	70
Cholesterol*	3% in benzene	25	24	+	0	0	0	0	5	16	21	53	63	74	80
,, *	,, ,, ,,	20	19	0	+	0	0	0	0	0	0	0	0	0	0
Benzene*	100%	25	24	+	0	0	0	0	0	6	30	45	50	50	55
Benzoyl peroxide*	1% in benzene	25	23	+	0	0	0	0	5	10	15	25	45	50	70
,, ,, *	,, ,, ,,	20	18	0	0	0	0	0	0	0	0	0	0	0	0
Hydrogen peroxide*	6% in 50% glycerol	25	20	+	0	0	0	0	6	6	17	33	42	47	58
,, ,, *	,, ,, ,, ,,	20	20	0	0	0	0	0	0	0	0	0	0	0	0
Glycerol*	100%	25	23	+	0	0	0	0	5	5	10	30	30	35	35

* To be compared with the second control series.

contained a group of non-treated irradiated controls, and the results of each group were then compared with its own control.

The animals were painted 3 times weekly, a camel's hair brush being used for the applications. The oils painted were wheat germ oil, cottonseed oil (Wesson oil), olive oil, linseed oil, and mineral oil. The wheat germ oil was a crude ether extract of wheat germ prepared in our laboratory. The other oils were purified oils as sold commercially, the mineral oil being of the grade used clinically. The peroxides applied were benzoyl peroxide‡ and hydrogen peroxide (Superoxal diluted with 50% glycerol to a 6% solution). The cholesterol‡ was recrystallized from alcohol. The carcinogenic chemicals were methylcholanthrene‡ and 1, 2, 5, 6 dibenzanthracene.‡ The concentrations of the various substances and the solvents used are given in Table I.

To determine whether the accelerating carcinogenic effect of the applied substances was a synergistic one, or due to the production of carcinogenic agents in the irradiated material, various oils and solutions were irradiated prior to application, and then painted onto the ears of non-irradiated animals. The solutions were irradiated in flat-bottom containers for one hour every week at a distance of 50 cm from an air-cooled Hg-vapor lamp. Solutions were prepared fresh every week. The animals were maintained on a diet of Purina dog chow and water *ad libitum,* and were examined every 2 weeks for neoplastic changes. Only definitely malignant growths were considered "tumors"; early thickenings and warts were classified as "intermediate". Results are expressed as the percentage of tumors at any given time—that is:

$$\frac{\text{(number with tumors)}}{\text{(number alive when first tumor develops)}} \times 100.$$

Most of the oils applied accelerated the rate of tumor production with ultraviolet light. Mineral oil was the most active, although cottonseed oil, olive oil, and wheat germ oil also had some effect. For example, after 4½ months of irradiation, tumors were present in 29% of the control animals, in 89% of those receiving mineral oil, in 56% of those receiving cottonseed oil or olive oil, and in 50% of those receiving wheat germ oil. Linseed oil retarded the production of ultraviolet tumors, probably due to the formation of a film of oxidized oil which decreased the penetration of the light. Glycerol also retarded tumor production somewhat. The stimulating action of oils on tumor production, therefore, did not appear to be due to a basic chemical property of the glyceride molecule, but rather to the physical characteristics of the oil—since mineral oil was the most active oil found. Mineral oil, however, did not appear to possess general carcinogenic properties. Irradiated mineral oil applied to non-irradiated animals failed to produce tumors and furthermore mineral oil failed to stimulate the production of tumors with methylcholanthrene. Methylcholanthrene was equally effective when painted in benzene or in cottonseed oil, but tumor production was actually retarded when it was applied in mineral oil. The stimulating action of mineral oil on tumor development, therefore, appears to be confined to ultraviolet tumors. The mechanism of this stimulation is unknown, but it is possibly due to a softening of the skin, permitting the penetration of more light to the active basal layer. It is also possible that the activity of the cells themselves is altered by the presence of oil.

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‡ Eastman Kodak Company.

Cholesterol markedly stimulated tumor development when applied in cottonseed oil but not when applied in benzene. Others have noted the formation of peroxides when cholesterol is irradiated in air[4, 5] and we have made a similar observation. However, the stimulating action of cholesterol on tumor development is probably not due to peroxides, since neither hydrogen peroxide, benzoyl peroxide, nor irradiated cholesterol were carcinogenic when applied to the ears of non-irradiated animals, nor did the presence of known peroxides accelerate the development of ultraviolet tumors. Furthermore, mineral oil, which accelerates the formation of ultraviolet tumors, does not develop peroxides on irradiation. It is possible that the extra amounts of cholesterol in the cottonseed oil enhanced the penetration of the oil into the skin, thus in effect increasing the action of the oil.

Our results with dibenzanthracene appear to be in line with those of Taussig, Cooper, and Seelig,[14] who found that ultraviolet light did not increase the carcinogenic activity of benzpyrene. However, they used relatively little light. It is of interest in this connection to note that dibenzanthracene, methyl cholanthrene, and benzpyrene all showed photodynamic activity when tested on *coleps*[15] indicating that all 3 are capable of producing biological changes in conjunction with light.

Summary. The rate of tumor production with ultraviolet light could be altered by the local application of certain substances to the tissues developing tumors. Of the oils tried, mineral oil accelerated tumor development most rapidly; cottonseed oil, olive oil, and wheat germ oil caused slight acceleration; linseed oil retarded tumor formation. Cholesterol in oil caused marked acceleration. Peroxides were without effect. 1,2,5,6, dibenzanthracene was also without effect.

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[15] Mottram, J. C., and Doniach, I., *Lancet*, 1938, **1**, 1156.

10958 P

Mechanism of Insulin Convulsions. III. Effects of Varying Partial Pressures of Atmospheric Gases After Adrenalectomy.

IRVINE MCQUARRIE, M. R. ZIEGLER, W. E. STONE, O. H. WANGENSTEEN AND C. DENNIS.

From the Departments of Pediatrics and Surgery, University of Minnesota, Minneapolis.

In a previous communication[1] on the effects of varying partial pressures of atmospheric O_2, N_2, and CO_2 on the blood sugar and serum electrolytes in the normal fasting dog, it was reported that breathing atmospheres containing between 5% and 12% O_2 caused marked hyperglycemia and a simultaneous reduction in the plasma K and inorganic P. Breathing a gaseous mixture containing 15% CO_2, 20% O_2, and 65% N_2 caused hyperglycemia accompanied by a marked rise in inorganic P but only a slight rise or no change in the K of the plasma. Convulsions either did not occur or their occurrence was greatly delayed when insulin was administered subcutaneously in doses of from 10 to 25 units per kilo of body weight (sufficient to cause convulsions in ordinary room air), if the animal was kept in the low O_2 or the high CO_2 atmospheres far beyond the periods previously found to be required for convulsions to occur. This effect was observed even when the blood sugar fell to levels well below those at which convulsions had occurred in room air. Breathing atmospheres containing from 60% to 80% O_2 had no detectable influence on the occurrence of insulin convulsions. Breathing an atmosphere of N_2 alone without administration of insulin caused convulsions within 2 minutes accompanied by a rise in K.

The present study was undertaken as an extension of the foregoing. A hope was entertained that any relationship that might exist between plasma electrolyte and blood sugar changes on the one hand and occurrence of convulsions on the other would be brought out more clearly in animals made highly sensitive to insulin by complete adrenalectomy. The effects of low O_2 (5% to 9%) and of high CO_2 (15%) were determined with and without administration of insulin. Methods used were the same as those described in the previous report. The effects of breathing low O_2 for from 2 to 3 hours were determined 6 times in 2 adrenalectomized dogs without administration of insulin. The response to breathing atmospheres containing 15% CO_2, 20% O_2, and 65% N_2 was determined 4 times in 2 adrenalectomized dogs before insulin was given. The effects of breathing an atmosphere of 5% O_2 and 95% N_2 on the response to insulin were determined 9 times in 5 adrenalectomized dogs.

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[1] McQuarrie, Irvine, and Ziegler, Mildred R., PROC. SOC. EXP. BIOL. AND MED., 1939, **39**, 525.

The results may be summarized as follows: Whereas breathing 5% O_2 plus 95% N_2 was found to cause a prompt and often fairly marked hyperglycemia in normal dogs, it was found to produce an equally prompt and sustained fall in the blood sugar in totally adrenalectomized animals. In contrast to the latter results, it was found that breathing a mixture of 15% CO_2, 20% O_2, and 65% N_2 caused a slight but significant rise of the blood sugar of the adrenalectomized dogs. While the anoxic anoxia of the first type of experiment caused definite decreases in the inorganic P and K, breathing 15% CO_2 resulted in a fairly marked increase in the P and a slight rise or no change at all in the K of the plasma.

As was to be expected, all of the dogs showed a marked increase in sensitivity to insulin following adrenalectomy, having convulsions after as little as 1% of the insulin dosages used before operation. The effect of breathing 5% O_2 plus 95% N_2 was to prevent the occurrence of convulsions completely in spite of the fact that the dose of insulin used was between 4 and 8 times the previously determined convulsive dose for a given adrenalectomized animal. The periods of observation under the low-O_2 experiments were approximately double those required to induce convulsions in room air. The degree of hypoglycemia was as great or greater at the end of the anoxic experiments as at the time of the convulsion in the control experiments. Blood glucose values as low as 9 to 14 mg % were recorded without the animal having convulsions. Breathing 15% CO_2, 20% O_2, and 65% N_2 had a similar effect so far as preventing convulsions was concerned. Blood sugar values decreased somewhat more slowly under the conditions of this experiment than under the previously described conditions, but fell to the previously determined level without the occurrence of a convulsion.

Conclusions. Anoxic anoxia (breathing 5% O_2 plus 95% N_2) causes hypoglycemia in adrenalectomized dogs without insulin in contrast to the hyperglycemia observed in normal animals. Insulin convulsions are prevented by anoxia of this degree even when the fall in blood sugar is maximal. Breathing 15% CO_2 likewise tends to prevent insulin convulsions from occurring.

10959

Active Immunity to Pneumococci in Rabbits Protected by Sulfapyridine.*

Milton Levine, W. P. Larson and Raymond N. Bieter.

From the Departments of Bacteriology and Pharmacology, University of Minnesota.

The results of experiments on the protection of rabbits, infected with pneumococci, by oral administration of sulfapyridine have been reported previously.[1] The purpose of this communication is to present data obtained in the study of the immunity in animals that had recovered from the infection produced under such circumstances.

Rabbits weighing approximately 2 kilos were inoculated intracutaneously with 0.3 cc of a 1/100 dilution of an 8-hour culture of a pneumococcus type II. The inoculum contained an average of 300,000 organisms as determined by plate-count. The virulence of the organism is such that an average of 1.5 organisms are necessary to cause an infection in 66% of a statistically sized group of mice weighing 20 g. This we have taken to be our minimal lethal dose. Its virulence for rabbits is such that 0.3 cc of a 1/1000 dilution of the original culture, containing 30,000 organisms, will kill all of the rabbits inoculated.

The drug-treated animals were given sulfapyridine orally in a 10% acacia suspension. Each animal received a total of 5 g of the drug, 0.5 g 1 hour before infection, 0.5 g 3 hours after the initial dose, and 0.5 g every 12 hours thereafter until the full dosage was administered.

Daily record was made of the temperature, and the size and nature of the skin-lesion. Rabbits recovering from the primary infection were reinoculated intracutaneously in the same general areas as previously, but not at the site of the original lesion if a scar had persisted. Only normal areas of the skin were used for reinoculation.

Type-specific Immunity. Twelve rabbits reinoculated intracutaneously with 0.3 cc of the whole 8-hour culture of the homologous type II pneumococcus 30 days after the original infection, showed no rise in temperature, and no local reaction around the point of inoculation. Five rabbits reinoculated 62 days after the original infection, and 5 after 83 days, showed no reaction. Of three rabbits

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* Aided by a grant from the Graduate Medical Research Fund. Merck & Co. kindly supplied the sulfapyridine used in these experiments.

[1] Larson, W. P., Bieter, R. N., and Levine, M., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 703.

reinoculated after 92 days, one showed a slight inflammation around the point of inoculation, while 2 showed no reaction. The rabbit with the local reaction ran a temperature for one day, then returned to normal. The controls (1 for each treated animal) all developed the typical lesions and died in from 4 to 10 days.

Six of the 12 animals given a second inoculation at the end of 30 days were injected a third time with the whole culture of the type II organism 94 days after the original infection, and again showed no reaction. These animals were reinoculated monthly thereafter, and at the end of 6 months showed no reaction to intracutaneous inoculation of the whole culture of the type II organism.

Species-specific Immunity. Eighteen of the animals recovered from the type II infection were inoculated intracutaneously with 0.3 cc of the whole 8-hour type II culture. This was done to raise the protective powers of the animals to a uniformly high level. There was no reaction to this inoculation. Ten days later these rabbits were reinoculated with 0.3 cc of the whole 8-hour culture of the type I pneumococcus; 15 controls were inoculated at the same time. The controls all died in from 5 to 10 days, showing typical lesions with edema, hemorrhage and inflammation. The virulence of the type I strain has not been determined, although the whole culture killed mice regularly on intraäbdominal injection, as well as the rabbits which were injected intracutaneously. The results of reinoculation of the 18 rabbits previously recovered from the type II infection are shown in Table I.

We next used a type III and a type VIII pneumococcus which were lethal for mice on intraäbdominal injection, but which produced a non-fatal infection in rabbits. Sixteen rabbits that had recovered from the type II infection were reinoculated intracutaneously with 0.3 cc of a whole 8-hour culture of a type III pneumococcus. Fourteen controls were inoculated at the same time. Twelve rabbits,

TABLE I.

Rabbits Recovered from Type II Infection and Reinoculated with Type I.

No. of rabbits	No. of days temp. above 103.5°	Type of skin lesion	Result
5	0	No reaction	Lived
4	1-3	Slight inflammation in the immediate area of inoculation	,,
3	4-5	More pronounced inflammation but no edema or hemorrhage	,,
2	6	Inflammation and edema	,,
4	Till time of death	Inflammation, edema and hemorrhage in the skin	Died

Of 18 rabbits, reinoculated, 14 lived; 15 controls all died.

which had recovered from a type II infection, and 12 normal controls, were inoculated with 0.3 cc of the whole 8-hour culture of the type VIII pneumococcus.

In both types, the control and reinoculated animals all lived. An examination of the lesions in the rabbits, as well as the temperature readings, revealed some striking differences in the reactions of the control and reinoculated groups. The lesions in the skin of the control groups were mild, showing little edema, but a widespread area of inflammation 3 to 4 inches in diameter. This inflammation persisted for from 5 to 7 days, then dried up leaving no scar. The lesions of the reinoculated group were localized at the point of inoculation, and presented on the second or third day a marked abscess-like induration which ulcerated, and in some cases formed a fistula through the skin so that the outer peritoneal wall was visible. No organisms could be recovered from these ulcers.

The temperatures obtained in these groups reveal that the infection persisted for a longer period of time in the control than in the treated animals, and this despite the formation in the reinoculated animals of ulcers which lasted in some cases as long as one month.

The average temperature of 18 rabbits recovered from infection with type II and then inoculated with type III was 105.7, 103.9, 103.3, and 102.9 on the 1st, 2d, 3d, and 4th days after inoculation. The averages for 15 control rabbits were 105.3, 104.2, 105.5, 105.1.

For 12 rabbits recovered from type II and then inoculated with type VIII the figures were 106.1, 103.8, 103.7, 103.6, 103.3, 102.8, 102.8, and for 12 control rabbits 105.7, 105, 105.7, 106.3, 105.2, 104.7, 103.7.

Two hundred readings on the rectal temperatures of normal rabbits indicate that 103.5°F is the upper limit of normal variation, and 102.4° the lower limit. Thus the temperatures of the control group inoculated with type III pneumococci remained up for more than 4 days whereas the reinoculated group showed a rise for one day and then a drop to about normal on the second day. Control rabbits inoculated with type VIII had abnormally high temperatures for 7 days, whereas the reinoculated group showed a drop on the second day, with an isolated rabbit having a temperature higher than normal on each of the following 3 days.

It is evident from the above data that in addition to a pronounced type-specific immunity present in rabbits recovered from type II infections, there is also a distinct species-specific immunity, as shown by the protective action against infection with the heterologous type I. The results obtained with the avirulent types III and VIII show a degree of protection due to the original type II infection, in that the lesions produced were limited in extent, and the temperatures of reinoculated animals were lower on the average than those of control animals. The ulcers formed may be indicative of an allergic condition set up coincident with a state of partial immunity.

10960 P

Conductive Cooling of Living Human Tissue.*

WILLIAM BIERMAN. (Introduced by U. J. Salmon.)

From the Department of Physical Therapy, Mount Sinai Hospital, New York City.

In the course of studies of temperature changes in the posterior male urethra, I investigated the effect of running cold water through a hollow metal rectal applicator. I found (as Table I indicates) that this procedure caused a definite fall in the temperature of the posterior urethra.

The posterior urethral temperatures were determined by means of a thermocouple (made of iron-constantan) with its tip protruding through an opening at the end of a silk woven catheter. The catheter was pushed beyond the cut-off muscle. The thermocouple was permitted to remain in the posterior urethra during the course of the experiment. The greatest fall in urethral temperature was 23.8°F, occurring in experiment No. 5. Before and after each experiment the accuracy of the thermocouple was checked by comparing its readings with standard thermometers.

Because of the marked effect of cold when applied conductively in this region, investigations of the influence of this measure were made in several other parts of the human body.

TABLE I.

Experiment No.	Urethral temperature °F		Temperature of water passing through rectal applicator	Duration of experiment	
	At start	At end		Hr.	Min.
1	99.1	95.2	70	1	15
2	99.1	93.3	62	1	10
3	99.	95.9	64		30
4	98.8	77.7	44	1	30
5	98.4	74.6	44	1	25

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* Aided by grants from the Council on Physical Therapy of the American Medical Association.

Ice bags were placed laterally on either side of the calf. An iron-constantan thermocouple (made by Leeds and Northrup) placed within a hollow needle 2 inches in length was inserted into the muscles of the calf. The thermocouple tip lay about 2 inches beneath the surface of that portion of the skin through which it was inserted and about 1½ inches away from either ice bag. The accuracy of this thermocouple was checked as previously described. The changes in the temperature of the calf muscle are shown in Table II. The greatest lowering of temperature (26.4°F) occurred in Experiment 1.

TABLE II.

Experiment No.	Temperature °F Initial	Temperature °F At end	Duration of experiment Hr.	Duration of experiment Min.
1	95.4	69.2	2	10
2	96.4	77.2	1	50
3	93.8	79.8	1	10
4	93.	78.	2	
5	92.	78.8	2	
6	97.4	77.	2	
7	97.	85.	1	30

A flat hollow metal applicator kept cold by circulating water through it (temperature 50-52°F) was placed on the outer side of the cheek. The temperature of the inner side of the cheek in the corresponding area was measured by means of an alcohol thermometer, with the lower portion of its stem bent at a right angle so as to permit good contact between its circular flattened base and the buccal mucosa. The changes in the temperature on the inner side of the cheek are shown in Table III.

TABLE III.

Experiment No.	Temperature Initial	Temperature At end	Duration, min.
1	96.	86.8	24
2	96.2	85.4	40
3	95.6	84.3	50
4	96.5	84.1	32
5	97.	85.5	32

A hollow metal cylindrical applicator was inserted into the vagina. It was cooled by circulating water through it. The temperature of the vaginal applicator and the changes produced within the rectum and urethra as determined by mercury thermometers are shown in Table IV.

In experiment No. 6, the urethral temperatures were observed for 10 minutes. The urethral thermometer was then pushed back into

TABLE IV.

Experiment No.	Vaginal applicator temperature		Rectal temperature		Urethral temperature		Duration	
	Initial	At end	Initial	At end	Initial	At end	Hr.	Min.
1	98.	52.	98.	73.5	98.	72.	1	
2	95.	52.5	99.	82.	97.5	90.5	1	25
3	90.	51.	97.	78.	97.	82.		30
4	98.	52.	98.	73.5	98.	93.	1	
5	93.5	47.5	96.5	73.	97.3	87.5	2	20
6	71.5	47.	99.2	70.	99.2	91.		50

the urinary bladder. During the subsequent 40 minutes the bladder temperature diminished from 91.8° to 86°F.

Conclusions. These observations show that it is possible to cause a substantial lowering of the temperature of the tissues of the living human body at a distance from a cold applicator placed in contact with the body surfaces. An explanation for this phenomenon is the occurrence of vasoconstriction and conductive cooling. These findings offer a basis for the therapeutic application of cold and indicate possibilities for its more extended use in the treatment of disease.

I desire to thank Drs. M. Friedlander, A. Hersh, F. T. Woodbury, and R. Walter for their assistance in the conduct of these studies.

10961

Blood Volume Changes in the Mammary Gland.*

J. C. SHAW AND W. E. PETERSEN.

From the Dairy Division, University of Minnesota.

In the studies of the metabolism of the mammary gland made by arteriovenous differences either no cognizance has been taken of blood volume changes or the data taken have been corrected on the basis of such changes. Blackwood and Stirling[1] were unable to demonstrate any blood volume changes in the mammary gland from their analyses of bloods for iron hemoglobin and cell volume. Lintzel[2] from determinations of hemoglobin by the Newcomer method observed no appreciable concentration changes between arterial and mammary venous bloods. Similarly Graham, Jones and Kay[3] from hemoglobin values obtained by the Palmer method reported that water losses to the mammary gland were insignificant. Graham, Peterson, Houchin and Turner,[4] however, observed blood volume changes by the use of Newcomer's method and corrected their data upon nitrogen partition of the blood accordingly but presented no data as to the magnitude of the blood volume changes.

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* Scientific Journal Series Paper No. 1759, Minnesota Agricultural Experiment Station. Prepared with the assistance of Work Projects Administration, Official Project No. 665-71-3-69. Sponsor: University of Minnesota.

[1] Blackwood, J. H., and Stirling, J. D., *Biochem. J.*, 1932, **26**, 357.

[2] Lintzel, W., *Z. Zucht.*, Reihe B., 1934, **29**, 219.

In a study of blood precursors of milk, hemoglobin determinations were made in triplicate by the method of Evelyn[5] using the Evelyn photoelectric colorimeter. Blood fats were determined by Allen's[6] method. The blood sugar was determined by the Shaffer and Somogyi[7] method. Large variations in blood volume were observed which will, in part, explain some of the conflicting results that have been reported.

Arterial and venous bloods were drawn simultaneously. The arterial blood was drawn in a manner similar to that described by Graham, Kay and McIntosh.[8] The venous blood was taken from the subcutaneous abdominal mammary vein. Errors due to dilution were avoided by drawing the same quantities of arterial and venous blood into vessels oxalated with identical quantities of potassium oxalate. During the drawing of the samples any excitation of the animal was noted and recorded. By using ethyl chloride to anesthetize the skin at the point of venipuncture we were able to obtain a large number of samples from a few animals without exciting them. Likewise, a large number of samples were drawn from animals which were in an excited state. Data are presented in Table I showing the results of this series of experiments. It will be observed that of the 23 experiments reported in which the animals were not excited the blood volume change exceeded 1% in only 3 cases. In 15 of the cases the blood volume change was less than 0.5%, which is within the error of the method. However, an entirely different picture is presented in those experiments in which the animals were

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[3] Graham, W. R., Jr., Jones, T. S. G., and Kay, H. D., *Proc. Roy. Soc. London*, Series B, 1936, **120**, 330.

[4] Graham, W. R., Jr., Peterson, V. E., Houchin, O. B., and Turner, C. W., *J. Biol. Chem.*, 1938, **122**, 275.

[5] Evelyn, K. A., and Salter, R. W., 1936, *Proc. Roy. Soc. Can.*

[6] Allen, N. N., *J. Dairy Sci.*, 1934, **17**, No. 5.

[7] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

[8] Graham, W. R., Jr., Kay, H. D., and McIntosh, R. A., *Proc. Roy. Soc. London*, Series B, 1936, **120**, 319.

TABLE I.
Effect of Excitation upon Blood Volume Changes in the Mammary Gland.

No Excitation			Slight or Marked Excitation		
Hemoglobin %		Venous blood volume change, %	Hemoglobin %		Venous blood volume change, %
Arterial	Venous		Arterial	Venous	
10.10	10.10	0.00	10.50	11.32	— 7.81
9.80	9.82	—0.20	10.33	10.82	— 4.74
8.30	8.30	0.00	9.24	9.55	— 3.35
8.23	8.23	0.00	7.80	7.96	— 2.05
8.65	8.63	+0.23	7.34	7.80	— 6.27
8.22	8.39	—2.07	11.40	10.49	+ 7.86
8.65	8.65	0.00	10.34	10.98	— 6.19
7.83	8.21	—4.85	8.39	9.61	— 4.54
8.94	8.94	0.00	6.13	6.44	— 5.06
9.55	9.55	0.00	11.35	11.80	— 3.96
8.55	8.62	—0.90	11.83	11.48	+ 3.89
5.95	5.95	0.00	9.55	9.86	— 3.24
10.77	10.84	—0.65	10.17	10.65	— 4.71
10.83	10.84	—0.09	10.57	9.55	+ 9.65
8.92	8.96	—0.45	8.22	8.36	— 1.70
10.25	10.49	—2.34	9.86	9.52	+ 3.45
10.82	10.78	+0.37	11.23	12.08	— 7.52
8.08	8.08	0.00	13.67	13.46	+ 1.54
9.86	9.86	0.00	12.08	13.83	—14.49
8.87	8.95	—0.90	13.67	14.33	— 4.83
9.55	9.55	0.00	11.15	11.48	— 2.06
7.45	7.52	—0.94	10.84	11.35	— 4.70
8.72	8.79	—0.80	12.48	13.41	— 7.45
Avg 9.00	9.07		10.33	10.70	
Mean deviation		0.643			5.70

either slightly or markedly excited. In all cases the arteriovenous blood volume change was considerably in excess of 1% with a mean deviation of 5.7.

In the majority of cases following excitement the venous blood was more concentrated than the arterial blood. It will also be observed that the average arterial hemoglobin values were higher following excitement than in those experiments where the animal was undisturbed. Due to the fact that the arterial blood concentration does vary more or less independently of the arteriovenous changes in concentration, it is apparent that when arterial and mammary venous bloods are drawn for milk secretion studies they should be taken simultaneously.

The question immediately arises as to what effect excitation and the resulting blood volume changes may have upon the arteriovenous differences of freely diffusible and non-diffusible substances. In the case of blood fats we have been unable to obtain a reasonable value in those cases where the arteriovenous differences were corrected for any considerable blood volume change.

The data presented in Table II represent an attempt to correct for

TABLE II.
Effect of Large Blood Volume Changes upon Loss of Blood Fat to the Mammary Gland as Determined by Arteriovenous Differences.

No change in blood volume	Dilution of venous blood			Concentration of venous blood		
Loss of fat to the gland mg%	Dilution %	Loss of fat to the gland mg%	Corrected loss of fat to the gland mg%	Concentration %	Loss of fat to the gland mg%	Corrected loss of fat to the gland mg%
11.90	7.14	—21.47	—34.61	14.54	35.60	65.00
10.07	9.65	5.25	—11.24	4.71	15.30	24.74
10.30	1.54	—13.9	—16.99	5.10	12.83	18.71
11.07	2.33	3.34	0.44	5.26	19.14	28.25
9.55	2.47	8.38	5.10	5.40	4.80	13.69
10.00	4.40	—18.89	—27.74	4.85	4.00	15.27
9.45	4.14	7.89	1.66	1.70	14.70	19.37
8.83	3.75	9.61	2.38	3.96	7.43	15.38
Avg 10.15	4.43	— 2.49	—10.13	5.69	14.23	25.05

such changes. All of these samples were taken 4 or more hours after milking. In those experiments in which no blood volume changes occurred there was a rather uniform loss of fat to the gland with an average loss of 10.15 mg %. Such was not the case, however, when there was any considerable arteriovenous blood volume change. The fat losses to the gland varied greatly with an extreme range of –21.47 to 35.60. When these values were corrected for blood volume change there was a much greater variation than before.

When the uncorrected and corrected data obtained in the dilution experiments are compared with similar data in the concentration experiments some striking results are noted which seem to offer some explanation for the large variations observed. It will be noted that with a dilution of the venous blood there is either a concentration of fat in the same blood as compared to the arterial blood or the loss of fat to the gland has been diminished. In the dilution experiments there was an average gain of 2.49 mg % of fat in the venous blood which when corrected for this dilution is increased to 10.13 mg %. However, when there was any considerable concentration of the venous blood over that of the arterial the blood fat was always lost to the gland and in quantities far in excess of what one would expect. Averages previously reported by Shaw and Petersen[9] as well as data published herein indicate that the average fat loss to the gland is 10 to 12 mg %. The average fat loss in this case of 14.23 mg % upon correction for concentration becomes 25.05 mg %.

It thus appears that when any considerable quantity of fluid

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[9] Shaw, J. C., and Petersen, W. E., *Am. J. Physiol.*, 1938, **123**, 183.

TABLE III.
Effect of Large Blood Volume Changes upon Loss of Blood Glucose to the Mammary Gland as Determined by Arteriovenous Differences.

	No blood volume changes	Large blood volume changes	
	Loss of blood glucose to the gland, mg%	Concentration or dilution (—), %	Loss of blood glucose to the gland, mg%
	11.4	11.20	10.0
	8.0	7.81	13.0
	12.6	4.74	9.0
	8.0	3.35	9.2
	10.0	6.27	12.4
	8.1	— 7.86	15.1
	11.6	— 7.14	13.5
	14.6	3.96	10.4
	12.6	4.71	16.6
	9.4	— 9.65	9.8
	16.0	3.45	16.8
	8.2	4.85	9.0
Avg	10.9		12.1
Mean deviation		6.25	

passes from the blood circulation to the gland or from the gland to the blood circulation it carries fat with it in varying amounts. Evidence is likewise accumulating showing that blood proteins are affected in very much the same way. An even more marked effect of disturbance of the animal than reported herein has been noted by Petersen and Shaw[10] in connection with arteriovenous carbon dioxide and oxygen ratios. Glucose on the other hand appears to pass in and out of the gland tissue with the water rather freely, as will be noted in Table III. The glucose differences reported in this table are similar to a much larger series of data previously reported by Shaw, Boyd and Petersen.[11] The apparent quantity of glucose lost to the gland in cases of blood volume changes does not differ materially from the quantity lost to the gland in those experiments in which blood volume changes do not occur.

Conclusions. 1. Large blood volume changes, not apparent in the undisturbed animal, occur in the mammary gland of the cow following excitation. 2. Arterial and mammary venous differences of nondiffusible blood substances are markedly affected by blood volume changes and cannot be corrected for on the basis of the observed changes in concentration.

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[10] Petersen, W. E., and Shaw, J. C., to be published.

[11] Shaw, J. C., Boyd, W. L., and Petersen, W. E., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 579.

10962

Factors Influencing Ovarian Response of Normal and Hypophysectomized Rat to Pregnant Mare Serum.*

Richard I. Pencharz.

From the Endocrine Laboratory, Department of Obstetrics and Gynecology, University of California Medical School, San Francisco.

Fluhmann[1, 2] states that the ovarian response produced by pregnant mare serum (PMS) is independent of the period over which the dose is distributed. He also adds that PMS is a less effective ovarian stimulant than either sheep anterior lobe or chorionic gonadotropic fractions presumably of equal potency. The former observation that the ovarian weight produced by a given level of PMS is independent of the time interval over which the dose is distributed has been confirmed, but the relative ineffectiveness of PMS was surprising in the extreme, and it is with this phase of the problem that the present communication is concerned.

Fluhmann used normal animals only and all injections were given subcutaneously.† There is considerable evidence that the method of injection may markedly influence the response to gonadotropic hormones. It is also now generally recognized that the pituitary of the intact animal may modify the ovarian response. For these reasons, one group of animals was injected subcutaneously and a second intraperitoneally. Both normal as well as hypophysectomized rats were tested.

One hundred and forty-five rats, 23 days old, of the Long-Evans strain, were divided into groups as indicated in Table I. The gonadotropic preparation used was Gonadin,‡ a commercial extract of PMS standardized to contain 200 R.U. (Cole-Saunders) in each cc of solution. Previous experience with this preparation showed that the actual unitage was higher than the labelled unitage. The stock solution, therefore, was restandardized on normal animals. One unit as here defined is the minimum amount of hormone, which when injected in a single dose intraperitoneally into immature rats

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*Supported by Christine Breon Fund for Medical Research.

[1] Fluhmann, C. F., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 313.

[2] Fluhmann, C. F., *Endocrinology*, 1939, **25**, 193.

† Personal communication after the present studies had been completed. In the original publications of Fluhmann, the mode of injection was inadvertently omitted.

‡ The pregnant mare serum preparation (Gonadin, lot No. x534) was generously supplied for this study through the courtesy of Mr. D. Wonder of the Cutter Laboratories, Berkeley, California.

TABLE I.

Effect of Dose, Subcutaneous and Intraperitoneal Injection on the Ovarian Response of Normal (23 days at onset of injection) and Hypophysectomized Rats (23 days at operation and 31 days at onset of injection) to Pregnant Mare Serum.

Group	No. of animals	Method of administration	Daily dose, R.U.	Total dose, R.U.	Avg wt of ovaries, mg	Avg wt of uterus, mg
I. Normal	3	Intrap.	2	6	73.2	202.0
	3	Subcut.	2	6	35.0	240.0
II. Hypd.	3	Intrap.	2	6	36.0	94.5
	3	Subcut.	2	6	17.5	28.5
III. Normal	36	Intrap.	2	12	180.3	203.7
	36	Subcut.	2	12	57.3	228.8
IV. Hypd.	15	Intrap.	2	12	226.6	150.4
	15	Subcut.	2	12	19.6	157.0
V. Normal	9	Intrap.	6	24	221.1	138.0
	9	Subcut.	6	24	237.1	138.0
VI. Hypd.	8	Intrap.	6	24	277.7	138.0
	5	Subcut.	6	24	250.0	136.0

23 days old, causes the formation of a small crop of medium large follicles and one to 2 small corpora lutea at the end of 96 hours. As an additional precaution to assure uniform dosage, the dilution of the daily dose was prepared from the restandardized solution just prior to injection to avoid loss of potency on standing.

The daily dose, contained in 0.25 cc, and given in a single injection, was 2 or 6 units. The periods of injection were 3, 4 and 12 days. Injection of the normal animals was started on the 23rd day of life. An interval of 8 days intervened between hypophysectomy and the onset of treatment in the operated groups. This was done to permit utilization of endogenous hypophyseal hormones circulating in the tissues at the time of hypophysectomy. Thirty-six hours after the last injection, the animals were sacrificed, the uteri and ovaries dissected free of extraneous tissues, weighed separately and fixed for subsequent studies.

The results secured by the 2 methods of injection are briefly summarized in Table I. Groups I and II served as pilot experiments and formed the basis for subsequent studies. It will be seen that subcutaneously administered PMS was a less effective ovarian stimulant in both normal and hypophysectomized rats. Intraperitoneally administered PMS was twice as effective as subcutaneous in both groups, but the response in the normal animal was twice as great as that secured in the hypophysectomized. The uterine weights were not significantly affected by the route of injection in the normal animal, but in the hypophysectomized intraperitoneal injection was approximately 4 times as effective as subcutaneous.

Decisive as these experiments are, it was felt, nevertheless, that the number of animals studied was too small to be crucial. Similar experiments, therefore, were repeated on larger groups injected over a more extended period of time. These disclosed the same enhanced effect with intraperitoneal administration. It will be noted that at a 24-unit level, 2 units on 12 successive days (Groups III and IV), the gravimetric ovarian response of the normal and hypophysectomized series injected intraperitoneally showed no significant difference. The ovaries of the 2 groups, normal and hypophysectomized, injected subcutaneously with the same total dose over a similar period of time, were markedly smaller than those treated intraperitoneally. In the normal animals the intraperitoneal route was approximately 3 times as effective as subcutaneous administration. The average ovarian weight of the former was 180.3 mg as compared with 57.3 mg in the latter. This difference in ovarian growth resulting from the 2 methods of treatment was still more striking in the hypophysectomized series. The average ovarian weight of the intraperitoneally injected rats was 222.6 mg as compared with 19.6 mg in the subcutaneously treated rats. Within each group, normal and hypophysectomized, receiving 2 units daily for 12 days, the uterine weights did not vary significantly with the method of treatment.

It must be admitted that the present work does not permit a direct comparison with that of Fluhmann. Fluhmann's preparation was standardized in Cutter units and the daily dose was given in 2 injections. The preparation used here was standardized somewhat differently and the daily dose was administered in a single injection. Nevertheless the data presented in this report would seem to offer a partial explanation for Fluhmann's findings in that they suggest that the apparent lack of effect of PMS which he reports might have been related to the route of administration.

While no complete explanation for the enhanced effect of intraperitoneal administration can be given at this time, the observation of Russel and Cori,[3] who found that the glycogenolytic effect of epinephrine is diminished in the hypophysectomized rat when given subcutaneously but not when administered intravenously, would seem to be relevant here. This they attribute to impaired subcutaneous absorption. It would seem likely, therefore, that the rate of absorption may also be important in determining the efficacy of PMS in the hypophysectomized rat, and to a lesser degree in the normal animal. The decreased subcutaneous effect in the hypophysectomized rat is not surprising, if it be remembered that hypophysectomy is followed by a lowered metabolic rate, a fall in blood pressure and diminished circulation.

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[3] Russel, J. A., and Cori, G. T., *Am. J. Phys.*, 1937, **119**, 167.

But the rate of absorption cannot be the only factor involved, for it will be seen in succeeding experiments that on augmentation of the daily dose subcutaneous administration was equally as effective as intraperitoneal.

In a recent paper, Evans, *et al.*,[4] state that the effectiveness of PMS is independent of the route of administration. The problem was, then, to reconcile the preceding results with the conclusion of Evans, *et al.* An answer to this was sought in groups V and VI listed in Table I. It will be noted that when the same total dose, namely 24 units, was distributed over a 4- instead of a 12-day period, subcutaneous administration was just as effective as intraperitoneal in both normal and hypophysectomized rats. The only outstanding difference between the 2 groups was the smaller uterine weights in animals injected over the shorter period. Fluhmann reports a similar progressive increase in uterine weight with PMS as the period of injection was prolonged.

At first sight it may seem that the results secured with groups V and VI contradict the thesis advanced earlier that subcutaneous administration is less effective than intraperitoneal. The paradox, however, is apparent rather than real. Other experiments now under way, and to be reported elsewhere, suggest that the effects produced by PMS depend on the size of the initial dose, whether it is given in a single or divided dose and on the route of administration. When relatively large doses are given, the discrepancy between subcutaneous and intraperitoneal injection is obscured. That this may indeed account for the conclusion of Evans, *et al.*, is strongly suggested by the present investigation.

Summary. A study of the ovarian response of the normal and hypophysectomized rat to PMS showed that subcutaneous administration is less effective than intraperitoneal. With small doses, 6 units distributed over 3 days and given at a 2-unit level in a single injection, the intraperitoneal route was twice as effective as subcutaneous in both normal and hypophysectomized. In the normal animal, PMS was twice as effective as in the hypophysectomized. The uterine response was not significantly affected by the mode of injection in the normal animal, but in the hypophysectomized intraperitoneal injection was 4 times as effective as subcutaneous. When the same daily dose is given for 12 days intraperitoneal injection was 3 times as effective as subcutaneous in the normal animal and 11 times as effective in the hypophysectomized. At this level, intraperitoneally administered PMS yielded ovaries of approximately the same weight in both groups. When the same total dose, namely 24 units, was distributed over a 4-day period, subcutaneous injection proved equally as effective as intraperitoneal in both normal and hypophysectomized rats. As a tentative explanation, it is suggested that the rate of absorption may be one of several factors involved in this mechanism.

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[4] Evans, H. M., *et al.*, *Univ. Calif. Pub. Anat.*, 1936, **1**, 237.

10963

Effect of A.T.10 (Dihydrotachysterol) on Various Types of Experimental Rickets in Rats.

ALFRED T. SHOHL, CH'UAN H. FAN AND SIDNEY FARBER.

From the Departments of Pediatrics and Pathology, Harvard Medical School, and the Infants' Hospital and the Children's Hospital, Boston.

A.T.10 (dihydrotachysterol) is, according to Holtz,[1] its discoverer, a reduction product of irradiated ergosterol. It has been claimed that it has a special capacity to raise the blood serum calcium concentration. Because of this remarkable property it has been used extensively in the treatment of parathyroid tetany. Albright, Bloomberg, Drake and Sulkowitch[2] have reviewed the literature and have studied the effects of this agent in comparison with vitamin D in cases of idiopathic hypoparathyroidism. They concluded that vitamin D has two fundamental actions: it increases the amount of calcium absorbed from the gastrointestinal tract and also the amount of phosphate excreted in the urine. A.T.10 acts similarly, but to a different degree; it has less effect on calcium absorption and greater effect on the excretion of phosphate in the urine.

A.T.10 has been reported not to cure experimental rickets in rats, except perhaps in toxic doses.[3] We have been unable to find a detailed account of Holtz's experiments on experimental rickets. We surmise that he used the classical high-calcium-low-phosphorus type of ricketogenic diets. If A.T.10 has the action described by Albright *et al.,* it is logical to postulate that it would be particularly unfavorable to the healing of rickets caused by low-phosphorus diets because it would increase the excretion of phosphate when phosphate must be conserved for deposition in bone. On the other hand, with a ricketogenic diet low in calcium and relatively high in phosphorus A.T.10 might be more effective because any increase in calcium absorption and any increase in phosphorus excretion would tend to alter conditions toward the normal. The present study concerns the problem of whether A.T.10 acts differently with various types of experimental rickets in rats, and not whether there is some slight antiricketic activity in the preparation, due either to a contaminant or to the A.T.10 itself.

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[1] Holtz, F., Gissel, H., and Rossmann, E., *Deutsche Z. f. Chir.*, 1934, **242**, 521.

[2] Albright, F., Bloomberg, E., Drake, T., and Sulkowitch, H. W., *J. Clin. Invest.*, 1938, **17**, 317; *Ibid.*, 1939, **18**, 165.

[3] Harnapp, G. O., *Monatschr. f. Kinderh.*, 1935, **63**, 262.

There is no good way of standardizing dihydrotachysterol in rat units, as is possible for vitamin D. Holtz has used as a unit the "toxische grenz Dose" (the minimal toxic dose), which he defines as the amount which, given daily, will cause a 15% loss of weight in 16-18 g male mice in 10 days, or the death of 50% of the animals. It is calculated that this minimal toxic dose contains 25μg (0.025 mg) of dihydrotachysterol.

The material used in this experiment was supplied by the Department of Medical Research of the Winthrop Chemical Co. It contained 200 minimal toxic doses per cc. Before feeding to the animals it was diluted with cottonseed oil which had been tested and found to contain no appreciable amount of vitamin D. The concentration was adjusted so that the desired dose could be given by putting one drop of the material directly into the mouth of the rat.

The rats were bred in our laboratory. The breeding animals were fed Sherman Diet B (⅔ wheat and ⅓ dried milk plus 1.3% of NaCl) and the young were weaned at 21 days of age and were continued on this diet until 28 days of age. They were then divided into 10 groups of 4 rats each and fed one of the 3 following basal diets: a normal diet (Sherman Diet B); the high-calcium-low-phosphorus ricketogenic diet No. 2965 of Steenbock and Black (79% ground whole yellow corn, 20% gluten and 1% NaCl, plus 3% $CaCO_3$); or a low-calcium ricketogenic diet (the same as diet No. 2965 with $CaCO_3$ omitted).[4] The calcium contents of these diets were, by analysis,[4] respectively, 0.33, 1.16, 0.016%; the phosphorus contents, 0.50, 0.30, and 0.32%. The Sherman Diet B had a Ca/P ratio of 0.66/1. Although the phosphorus contents of the 2 ricketogenic diets were identical, it is proper to speak of the Steenbock diet as a high-calcium-low-phosphorus diet because the Ca/P ratio was 4.0/1, and of the other as a low-calcium-high-phosphorus diet because the Ca/P ratio was 0.05/1. Group 1 received the normal

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[4] Shohl, A. T., and Wolbach, S. B., *J. Nutrition*, 1936, **11**, 275.

TABLE I.

Group	Basal diet	Daily supplement	Avg gain in body wt, 21 days, g	Ash of extracted femur, %	Blood serum		Evidence of rickets*	
					Ca, mg/100 cc	Phosphate, mg of P/100 cc	X-ray	Histology
1	Normal	—	55	56.3	11.4	8.9	—	—
2	High-Ca-low-P	—	17	35.7	10.3	2.9	++++	++++
3	,, ,,	Cod liver oil†	14	45.7	10.6	6.5	—	—
4	,, ,,	A.T.10, 1/8‡	18	41.0	11.3	4.4	++	++++
5	Low-Ca-high-P	—	5	38.9	6.4	8.3	—	+++
6	,, ,,	Cod liver oil†	20	42.3	8.5	8.1	++	±
7	,, ,,	A.T.10, 1/800‡	6	39.4	7.3	7.9	—	+++
8	,, ,,	A.T. 10, 1/80‡	5	37.6	7.0	9.2	—	±
9	,, ,,	A.T.10, 1/8‡	12	41.9	5.9	10.8	—	—
10§	,, ,,	A.T.10, 1/8‡	3	39.9	5.1	8.5	—	+

* — = no rickets; ± = borderline rickets; + = rickets. The degree of rickets is indicated by the number of plus signs, from one, which is mild, to 4, which is most severe rickets.

† One drop of cod liver oil, equivalent to 9 units of vitamin D.

‡ The fractions are in terms of the "minimal toxic dose."

§ The supplement was added to the basal diet of this group at the end of 21 days, and the experiment continued for 7 days longer.

diet and served as a normal control. Groups 2, 3 and 4 were given the high-calcium-low-phosphorus ricketogenic diet; group 3 received a supplement of cod liver oil and group 4 of A.T.10. Groups 5, 6, 7, 8 and 9 were fed the low-calcium-high-phosphorus ricketogenic diet; group 6 received a supplement of cod liver oil equivalent to 9 units of vitamin D; and groups 7, 8 and 9, three different amounts of A.T.10 respectively. All supplements were given daily. The duration of this experiment was 21 days. This procedure constitutes a test of protection against development of rickets. The rats of group 10 were fed the low-calcium-high-phosphorus rickets-producing diet for 21 days and then given a supplement of A.T.10 for 7 days. The purpose of this experiment was to test the curative effect of A.T.10.

At the end of the experiments the animals were X-rayed, weighed and bled from the femoral artery under ether anesthesia. The blood serum was analyzed for calcium and inorganic phosphate. The bones of one leg were preserved in 10% formalin for histologic examination; those of the other were used for determinations of the percentage ash of the dried bones after extraction with alcohol and ether. The methods of analysis used have been previously described.[4] The relevant data are presented in Table I.

Results. The criteria for interpretation of both roentgenograms and microscopic anatomy have been described in detail in a previous study.[4] The rats fed the high-calcium-low-phosphorus diet alone (group 2) showed the usual classical findings of rickets both by X-ray and by histologic examination of decalcified sections of the bones. The rats fed the same diet plus cod liver oil (group 3) showed complete protection by both methods of examination. The rats fed the same diet plus A.T.10 (group 4) were not protected by even the largest dosage used in this study.

The X-ray pictures of the bones of the low-calcium-high-phosphorus groups are very difficult if not impossible to interpret, because this type of diet results in bones with a very narrow zone of cartilage. The diagnosis of rickets depends upon interpretation of the histology of bone. When so examined, the bones of rats which were fed low-calcium-high-phosphorus diets in group 5 (without supplement) showed marked rickets; those in group 6 (with cod liver oil) showed little or no rickets; and those in groups 7, 8 and 9 showed progressively less rickets as the dose of A.T.10 was increased, and absence of rickets when ⅛ minimal toxic dose was given.

When A.T.10 was administered after low-calcium-high-phosphorus rickets had developed (group 10), the healing was far advanced after 7 days' administration. Thus the evidence for protection and cure both show the effectiveness of A.T.10 as an antiricketic agent in this type of rickets.

The animals fed the low-calcium-high-phosphorus diets, except those receiving cod liver oil, seemed more lethargic than normal animals or even than those which were given the high-calcium-low-phosphorus ricketogenic diets.

The average gains in weight of rats fed the ricketogenic diets, especially the low-calcium-high-phosphorus diet, were far less than those of the normal controls. Neither cod liver oil nor A.T.10 effected increases with the high-calcium-low-phosphorus ricketogenic diet. Both caused gains in weight with the low-calcium-high-phosphorus diet; the gains with A.T.10 were greatest when the amount given afforded complete protection against rickets.

In the dosages used the effects of A.T.10 on the concentrations of calcium and inorganic phosphate in the blood serum were not striking. Whatever may be its action in raising serum calcium after removal of the parathyroid glands, A.T.10 did not, under the given conditions, cause an increase of calcium concentration in the blood serum. It caused a slight increase in the serum phosphate in high-calcium-low-phosphorus rickets. The usual values for calcium and phosphate were found in the controls.

When protection against the development of rickets with A.T.10 was accomplished, it occurred without significant increase in either the absolute amount or percentage of ash, water, or organic matter in the femurs. This is in contrast to the effect of vitamin D on high-calcium-low-phosphorus rickets in which deposition of minerals is marked. When cure was initiated with A.T.10 a large increase in the amount of fat occurred (not shown in table).

The data show that in the dosage used the effects of A.T.10 can be interpreted only by a study of the bone histology; the roentgenograms, blood serum and bone ash show but slight changes from the normal. The histologic studies are, however, conclusive. They demonstrate that the action of A.T.10 can be sharply differentiated from that of cod liver oil. The latter is effective in the prevention and cure of rickets with both types of diets, but an amount of A.T.10 which is without demonstrable effect in prevention of rickets in rats on the usual high-calcium-low-phosphorus diet, protects them from developing rickets when they are fed a low-calcium-high-phosphorus diet. Even if the slight increase in serum phosphate and modification of rickets as shown by X-ray are attributed to an antiricketic action of A.T.10 upon rats fed the high-calcium-low-phosphorus type of diet, this effect is much less marked than upon those fed the low-calcium-high-phosphorus diet. Therefore, even if the material used is contaminated with vitamin D, or in itself has antiricketic properties when used in massive doses, the difference in its action upon the two types of rickets is manifest. Although the exact composition of the preparation is not known, it is obvious that it does not contain a large amount of vitamin D, for it did not cure rickets in rats resulting from high-calcium-low-phosphorus intake, even with the largest doses used. Furthermore, the cure of the rickets in rats resulting from low-calcium-high-phosphorus diets was not effected by the toxic factor, for the blood serum calcium was not raised nor was pathological calcification produced in the soft tissues. It is reasonable to conclude, as do Albright *et al.,* that A.T.10 and vitamin D affect calcium and phosphorus metabolism to a different degree, and that A.T.10 is more effective in preventing rickets induced by the low-calcium-high-phosphorus diet because it facilitates the elimination of phosphate in the urine and hence renders this type of diet less ricketogenic.

Summary. The effect of A.T.10 on prevention of rickets in rats differs according to the ricketogenic diet fed. Rickets caused by high-calcium-low-phosphorus diets was not prevented by a non-toxic amount of A.T.10 which protected rats fed low-calcium-high-phosphorus diets.

10964

Resistance of Young Dogs to Acute Arrest of the Cephalic Circulation.*†

HERMAN KABAT AND CLARENCE DENNIS. (Introduced by M. B. Visscher.)

From the Department of Physiology, University of Minnesota.

The observation has been made by several investigators that the young animal is much less susceptible to asphyxia than the adult.[1, 2]

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† Assistance in the preparation of these materials was furnished by the personnel of Works Progress Administration, Official Project No. 665-71-3-69, subproject No. 309.

[1] Reiss, M., *Z. f. d. ges. exp. Med.*, 1931, **79**, 345.

[2] Avery, R. C., and Johlin, J. M., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1184.

No information has been available, however, concerning the essential factors involved in the greater resistance of the young to asphyxia. It was of interest, therefore, to determine whether the brain itself is more resistant to anoxia and accumulation of metabolites in the young animal. This question has been investigated by comparing the effects of complete arrest of the cephalic circulation for various periods of time in adult dogs and in puppies. The method which has been employed to produce sudden complete stasis of blood in the head by means of a pressure cuff in the cervical region has been described[3] as has also the effects of this procedure on adult animals.[4]

The adult dog rarely recovers consciousness after 8 minutes of complete vascular occlusion. It remains comatose as long as it lives, losing all sensation and retaining only very primitive responses on the reflex level. After 6 minutes of vascular stasis, consciousness is recovered in 24 to 48 hours as a rule and following this, the animal is very severely ataxic, restoration to normal requiring a period of weeks or months. Following 4 minutes of arrest of the cephalic circulation, consciousness returns in 18 to 24 hours and the residual ataxic symptoms, though somewhat less severe, are still present. After only 2 minutes of vascular occlusion, slight ataxia persists for a week or more.

Complete arrest of the cephalic circulation was produced in 8 puppies using the same technic as had been employed in the study of adult animals. The results of temporary cessation of the brain circulation in these animals are in striking contrast to the effects of the procedure in the adult. Various responses such as the wink reflex, respiration, struggling of the body and movements of the tail all persist longer after arrest of cephalic blood flow in the young animal. Thus the lid wink persisted for one minute and in some cases for 1½ minutes after vascular occlusion in the puppy, while it disappeared in 10 to 20 seconds in the adult. Similarly, gasping continued for 2½ minutes as a rule, while in the adult all respiration had ceased less than one minute after circulatory arrest. Movements of the legs and tail continued for an average of 6 minutes in the puppy, while in the adult, movements had ceased within 2 minutes. However, there is little reason to doubt that the arrest of circulation effected by the procedure is complete, since after an arrest of cephalic circulation for 10 to 12 minutes, the wink reflex did not return for 8 to 10 minutes following restoration of blood flow.

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[3] Kabat, Herman, and Dennis, Clarence, Proc. Soc. Exp. Biol. and Med., 1938, **38**, 864.

[4] Dennis, Clarence, and Kabat, Herman, Proc. Soc. Exp. Biol. and Med., 1939, **40**, 559.

Six of the puppies were littermates, weighing 2 pounds, all of whom were studied on the same day at the age of 45 days. Following 4 minutes of circulatory stasis, a puppy could stand and walk with only moderate ataxia within 20 minutes. After 1½ hours, the animal was quite responsive and could walk without ataxia, and its behavior differed very little from that preceding the arrest of blood flow.

In another animal, 4 hours after complete circulatory arrest of 12 minutes' duration, the puppy was able to stand and walk very ataxically, walking on a broad base with the nose close to the ground. It responded sluggishly to sound and light and was very hyperactive, walking all of the time. After 24 hours, the puppy was unable to stand or even to turn from its side to its belly, although it was able to lap milk, see, and hear. It gradually improved, so that on the third day following the arrest of the cephalic circulation, the animal was able to stand and walk but very ataxically, falling frequently. Much cerebral function was retained, since the puppy responded to petting by wagging its tail, came when he was called and walked across the room for his milk, etc. At 10 days, the chief symptom was a moderately severe ataxia with an intention tremor of the head.

In another animal, 16 minutes of cephalic vascular stasis resulted in coma which persisted. The animal never regained consciousness or sensations and only primitive reflex responses remained.

In order to examine more closely the age factor in the resistance to arrest of the cephalic circulation, 2 littermate puppies were subjected to circulatory arrest of 10 minutes' duration, one at the age of 48 days and the other a month later at the age of 77 days. The younger animal was able to see and hear, stand and crawl forward ataxically 2 hours after the procedure. The next day it showed only a slight ataxia, from which it recovered completely by the second day, at which time its behavior was quite normal.

The older littermate was much more severely affected by the 10 minutes of circulatory arrest in the brain. After 6 hours, it was semi-comatose, gave evidence of ability to see and hear, and was hyperactive, crawling forward very ataxically. On the following day, the puppy could not stand or even turn from its side to its belly and gave no response to sound or light. It lapped when its mouth was placed in milk. After 4 days, sensation had been restored and the puppy could turn from its side to its belly but was unable to stand or walk. On the fifth day, it could stand and walk very ataxically, came when it was called and was curious about its environment. It is interesting to note that in this case as well as in the 12-minute occlusion described above, recovery had proceeded much further within a few hours after resuscitation than on the following day. This may indicate that a secondary depressive factor acts on the neurons some time after the circulatory arrest.

It is evident from these results that the brain of the young dog is much more resistant to arrest of its circulation than is that of the adult dog and this increased resistance appears to bear a reciprocal relation to the age of the animal. The increased resistance of the young brain to circulatory arrest may correlate with a lower oxygen requirement of the neurons, since Himwich, Baker and Fazekas[5] have demonstrated that the oxygen consumption of slices of infant rat brain is much smaller per unit wet weight than that of the adult and Craigie[6] has shown that the infant brain is considerably less vascular than that of the adult.

10965 P

Effect of Hypothermia on Cerebral Metabolism.

JOSEPH F. FAZEKAS AND HAROLD E. HIMWICH.

Department of Physiology and Pharmacology, Albany Medical College, Union University, Albany, N. Y.

We have previously presented data[1] indicating an increased cerebral metabolism during hyperthermia. In this communication are included the changes of cerebral A:V oxygen differences resulting from hypothermia. The recent work of Smith and Fay[2] in which hypothermia has been used for the treatment of cancer lends added significance to this study.

Dogs anesthetized with pentobarbital were packed in ice. Blood samples were collected from the femoral artery and the superior longitudinal sinus at various intervals while the rectal temperature fell from approximately 38°C to 26°C. The blood samples were analyzed for oxygen[3] and glucose.[4] The velocity of the systemic circulation was estimated by the method of Robb and Weiss.[5] Nine dogs have

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[5] Himwich, H. E., Baker, Z., and Fazekas, J. F., *Am. J. Physiol.*, 1939, **125**, 601.

[6] Craigie, E. Horne, *J. Comp. Neurol.*, 1925, **39**, 301.

[1] Himwich, H. E., Bowman, K. M., Goldfarb, W., and Fazekas, J. F., *Science*, 1939, **90**.

[2] Smith, L. W., and Fay, T., *J.A.M.A.*, 1939, **113**, 653.

[3] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[4] Hagedorn, H. E., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 46.

[5] Robb, G. P., and Weiss, S., *Am. Heart J.*, 1932-3, **8**, 650.

TABLE I.

Time	Temp.	Oxygen Vol. % A	Oxygen Vol. % V	Diff.	Sugar Mg % A	Sugar Mg % V	Diff.	Circulation Time, sec	Heart Rate	Resp.
9:05	39.5	18.15	9.81	8.34	88	84	4	7	180	
9:34	39.5	18.24	10.59	7.65	99	83	16	6	170	16
10:25	33	18.33	13.13	5.20	95	86	9	12	108	
10:45	29	19.19	17.42	1.77	125	122	3	44?	50	1

been thus examined. The results have been similar in all instances and a typical experiment is therefore presented in Table I.

It may be observed that despite a slower circulation as evidenced by a decreased heart rate and prolonged circulation time the cerebral A:V difference diminishes as the rectal temperature falls. A slower circulation rate by itself would make for a greater A:V difference with a constant cerebral metabolic rate. Obviously, then, there must have been a great diminution of cerebral metabolism as a result of the fall of temperature. The similarity of these results with those obtained during insulin hypoglycemia is striking.[6]

Summary. Nine dogs, subjected to hypothermia, suffered a decreased cerebral metabolism as indicated by a smaller A:V difference despite a slower blood flow.

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[6] Himwich, H. E., Bowman, K. M., Wortis, J., and Fazekas, J. F., *J. Nerv. and Ment. Dis.*, 1939, **89**, 273.

10966

Effect of Sulfapyridine on Immune Response to Pneumococcal Infection.*

JOSEPH C. EDWARDS, THEODOR E. KIRCHER, JR., AND LAWRENCE D. THOMPSON. (Introduced by D. P. Barr.)

From the Department of Medicine, Washington University, St. Louis, Mo.

With the introduction of sulfapyridine-therapy in the pneumonias, numerous studies have appeared on the mode of action of the drug. In the hope of obtaining some evidence concerning its effect upon immune responses to the pneumococcus, we undertook the following study in patients who were treated in the hospitals of St. Louis during the year 1938-39.

To date there are in the literature very few data on this subject. Larson[1] and his associates found that mice, recovering from pneumococcal infection after treatment with sulfapyridine, developed no signs of reinfection after reinoculation with homologous type-specific (type 2) pneumococci. If the virulence of the particular strain used is great, the mice or rabbits may die before sulfapyridine inhibits the organisms.[2] A similar quantity of killed pneumococci gives the same protection against reinfection, and sulfapyridine adds nothing to this protection. This work would indicate, however, that sulfapyridine does not interfere with the usual processes of immunity.

Three groups of patients were studied to determine the occurrence of type-specific and species-specific agglutinins, heterophile antibodies, and dermal reactivity to type-specific polysaccharides. Twenty-six patients with pneumonia treated with sulfapyridine were subjected to all the tests before, during, and after their illness and 40 additional patients were tested for heterophile antibodies before and after sulfapyridine-therapy. For comparison, and in a measure as controls, data were gathered concerning 16 patients who were completely tested for agglutinins, heterophile antibodies, and dermal response before and after treatment with specific antipneumococcal serum, as well as 80 similarly treated patients in whom heterophile antibodies only were determined. The same studies were performed on serum from 2 patients who were treated with sulfapyridine and type-specific serum.

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* This work was made possible through the generosity of the Eli Lilly Company, Indianapolis, Ind. We wish to thank H. M. Powell and W. A. Jamieson for their cooperation in the determination of heterophile antibody. Dr. Avery Rowlette, Dr. S. H. Gray, and Dr. George Hunt of St. Louis City Hospital also gave splendid cooperation; Dr. David P. Barr and Dr. L. A. Julianelle gave helpful criticism in the final preparation of the paper.

[1] Larson, W. P., Bitter, R. N., and Levine, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 703.

[2] Long, P. H., *J. A. M. A.*, 1939, **112**, 1850.

Blood was obtained before, during (at or soon after the temperature became normal) and after treatment. In several cases specimens were obtained daily or on alternate days during hospitalization.

Agglutinations were done with suspensions of formalinized pneumococci in the usual dilutions of serum. Observations were recorded at the end of one hour and again after 24 hours at 37°C. Types 1, 2, 3, 7, and 8 pneumococci were checked for type-specificity by the Neufeld *Quellung* technic and tested for titer with homologous antipneumococcal rabbit serum. In addition to the above types, we used a rough strain of Type 2† grown on nutrient broth 8 to 12 hours and preserved with 0.2% formalin.

Skin-testing with type-specific capsular material was done as described by Francis[3] and ourselves.[4]

Heterophile antibody was titrated with defibrinated sheep-blood.

In general the titers of type-specific agglutinins following sulfapyridine-therapy did not increase either before or during defervescence, which usually occurred within 48 hours unless pneumonia was then in its sixth to eighth day, at which time an increase in type-specific agglutinins normally appears. Following intravenous type-specific serum there is, of course, an increase in type-specific agglutinins in proportion to the excess of serum given, with little or no effect on the agglutinins for the rough or species-specific pneumococcus. Sulfapyridine-treated patients, as a rule, exhibited species-specific agglutinins earlier and in a higher titer than they did type-specific agglutinins.

The skin reaction to polysaccharide was found to be a reliable guide to proper dosage of type-specific serum, being positive in the presence of an appreciable titer of type-specific agglutinins. Following sulfapyridine-therapy alone, the skin-reaction became positive again only when type-specific agglutinins were present in sufficient amount. There was no direct correlation between fall in temperature and the type-specific antibody-titer in the sulfapyridine-treated cases.

The heterophile antibody seemed to have no constant relation to the course or outcome of the pneumonia nor to the type or severity of any particular case, nor to the type of therapy, whether with serum or drug. Its titer did not parallel that of either the type-specific or species-specific agglutinins. Incidentally, we found, as have others,[5] no indication that the heterophile titer was directly related to or responsible for serum-sickness. There was no correlation between the heterophile titer and the positivity or negativity of the cutaneous reaction to polysaccharide. There was some increase in its titer following the administration of species-specific serum high in heterophile-antibody content.

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† Obtained from Dr. Marian Morris, Department of Bacteriology, Washington University School of Medicine.

[3] Francis, T., Jr., *J. Exp. Med.*, 1933, **57**, 617.

[4] Edwards, J. C., Hoagland, C. L., and Thompson, L. D. Read at General Scientific Session of A.M.A. Meeting in St. Louis, May 15, 1939. Accepted for publication in *J. A. M. A.*

Summary. 1. The immune responses that occur naturally in the course of an untreated pneumonia, studied by determining type-specific and species-specific agglutinins and by the dermal reactivity to type-specific polysaccharides, are apparently unaltered by treatment with sulfapyridine. 2. There seems to be no relation of the heterophile-antibody titer to the clinical course of pneumococcal pneumonia or its immune responses following sulfapyridine-therapy.

10967

Toxicity of the Ortho, Meta and Para Isomers of Sulfanilamide.

EDWIN P. LAUG AND HERMAN J. MORRIS (Introduced by Erwin E. Nelson.)

From the Division of Pharmacology, Food and Drug Administration, U. S. Department of Agriculture, Washington, D. C.

While the therapeutic potency of p-sulfanilamide has been amply demonstrated, reports by a group of French investigators[1] indicate that the protective action of the ortho and meta isomers against experimental infection in mice is practically nil. The present investigation was undertaken to determine whether there is any corresponding difference in the toxicity of the 3 isomers.

The acute toxic effects for mice were determined by oral administration of the isomers in 33% suspension in 5% gum acacia to fasted animals. The manifestations of toxic action were quite different among the 3 isomers. When the para-isomer was given there usually resulted, in from 30 minutes to an hour, a preliminary stage of stimulation, with muscular incoordination and spastic paralysis of the hind limbs. This was followed by coma, sometimes lasting 18 to 24 hours, and death. After the ortho isomer, however, there was no evidence of either stimulation or spastic paralysis. Instead the animals became comatose within 30 minutes, and died after 18 to 24 hours, with no additional symptoms other than slow and labored respiration. The above effects were induced by a dosage level of from 4 to 6 g per kilo for both ortho and para isomers. The meta isomer was without effect at this dosage, but at 10 g per kilo there were symptoms suggestive of the action of para isomer, but with prompt recovery.

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[5] Powell, H. M., Jamieson, W. A., and Kempf, J. F., *J. Immunol.*, 1935, **29**, 267.

[1] Trefouel, J., Trefouel, J., Niti, F., and Bovet, D., *Ann. Inst. Pasteur,* 1937, **58**, 30.

The results of the toxicity studies have been tabulated in Table I. Calculation of the LD50's and the regression coefficients from the 3 respective dosage-mortality curves were obtained by treating the data according to the method outlined by Bliss[2] and described by us[3] in detail elsewhere. The ascending order of toxicity is meta, para and ortho, and it should be noted that the differences between the respective LD50's are significant. The toxicity of the meta isomer proved to be so low that sufficient quantities could not be administered to effect even 50% mortality without danger of mechanical injury to the animals. Consequently the LD50 value had to be ob-

TABLE I.
Acute Toxicity of Ortho, Meta, and Para Isomers of Sulfanilamide for Mice.

	Dose, g/kg	Mortality Ratio No. mice died / No. mice used	LD50 g/kg	Fiducial* limits	Regression Coefficient	Fiducial* limits
	2.5	1/10				
	3.0	1/10		4.15		11.5
Ortho	3.5	2/10	3.84		8.58	
	4.0	16/25		3.55		5.65
	5.0	22/25				
	6.0	23/25				
	4.0	1/25				
	5.0	3/25		19.3		6.06
Meta	6.0	3/25	12.5		3.36	
	7.5	2/10		8.04		0.65
	10.0	4/10				
	3.5	0/10				
	4.0	8/25		4.86		15.4
Para	4.5	5/10	4.61		11.5	
	5.0	24/40		4.39		7.54
	5.5	7/10				
	6.0	24/25				

* For odds of 19 out of 20 trials.

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[2] Bliss, C. I., *Quart. J. Pharm. and Pharmacol.*, 1938, **11**, 192.

[3] Laug, E. P., Calvery, H. O., Morris, H. J., and Woodard, G., *J. Ind. Hyg. and Toxicol.*, 1939, **21**, 173.

TABLE II.
Solubility of Ortho, Meta and Para Isomers of Sulfanilamide at 28°C and 38°C.

	Grams dissolved per 100 g H_2O	
	28°C	38°C
Ortho	0.808	1.317
Meta	1.508	2.796
Para	0.940	1.731

tained by interpolation of the rectified mortality curve. This accounts in part for the wide limits of error for the value given. In the case of the regression coefficients, there is a significant difference between meta and para only. Thus, it would appear that the observed toxicities show no correlation with the reported therapeutic potencies.

In Table II are given the results of solubility measurements at 28° and 38°. These represent averages of closely agreeing triplicate determinations. While it could be expected that solubility might modify the toxicity of a compound, no correlation was noted in the case of the isomers. M-sulfanilamide, roughly twice as soluble as either o- or p-, is the least toxic, while o-sulfanilamide, the least soluble, is the most toxic. Thus it would appear that solubility, in so far as it may influence absorption from the gut, is not a factor to be considered in the toxicity of these three isomers.

Dyke[4] has reported on the acute toxicity of the 3 isomers for mice with LD50's ranging from 3 to 5 g per kilo. However, examination of the data reveals considerable scattering of the results, as well as the use of restricted numbers of animals. No statistical analyses of the results, nor exact calculation of LD50's were attempted. For p-sulfanilamide alone, Marshall and coworkers[5] give 3.8 g per kilo, Wien,[6] 4.2 g per kilo. Our value, 4.61 g per kilo is somewhat higher.

Conclusions. (1) The LD50's for oral administration to mice of the isomers of sulfanilamide are: ortho, 3.84 g; meta, 12.45 g; para, 4.61 g per kilo. (2) There is no correlation between solubility and toxicity of the 3 isomers. (3) There is no relation between toxicity as here found and therapeutic efficiency as reported by others.

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[4] Dyke, W. J. C., *Chemist and Druggist*, 1937, **127**, 126; *Quart. J. Pharm. and Pharmacol.*, 1937, **10**, 319.

[5] Marshall, E. K., Cutting, W. C., and Emerson, K., *J. A. M. A.*, 1938, **110**, 252.

[6] Wien, R., *Quart. J. Pharm. and Pharmacol.*, 1938, **11**, 217.

10968 P

Influence of Anti-Anemic Principles upon Size of Erythrocytes of Opossums (*Didelphis virginiana*) in Maternal Pouch.

Joseph Stasney and Edward L. Burns (Introduced by Kenneth L. Burdon.)

From the Department of Pathology and Bacteriology, School of Medicine, Louisiana State University, New Orleans.

The red blood cells of the mammalian fetus are constantly larger than those of adults of the same species, and resemble very closely the erythrocytes found in individuals with pernicious anemia.[1] It has likewise been shown experimentally that the mean maximal diameter of erythrocytes of newly born rats can be reduced at birth by the administration of concentrated normal gastric juice to the mother during pregnancy.[2]

The inaccessibility of common mammalian fetuses to direct injection made it desirable to devise a scheme by which anti-anemic principles could be injected directly into the developing animals themselves. The opossum *(Didelphis virginiana)* is suitable for this purpose because intrauterine life lasts only 10.5 ± 1.7 days, after which the immature young live in the maternal pouch for 90 days.[3]

Materials and Methods. Forty-nine immature opossums, varying in crown-rump lengths from 5.5 to 7.0 cm, were used in these experiments. Twenty were injected over periods varying from 3 to 13 days with 0.8 to 2.2 cc of concentrated normal human gastric juice. Ten untreated pouch mates served as controls. Nine were injected over periods varying from 3 to 14 days with from 0.3 to 2.6 cc of concentrated solution of liver extract. Ten untreated litter

TABLE I.
Comparison of Mean Maximal Diameter and of Mean Corpuscular Volume of Erythrocytes of Immature and Adult Untreated Opossums.

	Untreated immature opossums	Untreated adult opossums
No. of animals	49	10
Mean max. diam. of erythrocytes in μ	8.90	7.30
Variance of means	0.00240	0.00200
No. of animals	39	10
Mean corpuscular vol. of erythrocytes in μ^3	162.71	88.49
Variance of means	2.90406	5.91789

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[1] Wintrobe, M. M., and Shumacker, H. B., *J. Clin. Invest.*, 1935, **14**, 837.

[2] Stasney, J., Higgins, G. M., and Mann, F. C., *Am. J. Med. Sci.*, 1939, **197**, 690.

[3] Hartman, C. G., *J. Morph. and Physiol.*, 1928, **46**, 143.

TABLE II.

Effect of Normal Human Gastric Juice and of Concentrated Liver Extract Solution on the Mean Maximal Diameter and Mean Corpuscular Volume of Erythrocytes of Immature Opossums.

	Gastric Juice				Liver Extract			
	Experimental animals		Control animals		Experimental animals		Control animals	
	Beginning of exp.	End of exp.	Beginning of exp.	End of exp.	Beginning of exp.	End of exp.	Beginning of exp.	End of exp.
No. of animals	20	20	10	10	9	9	10	10
Mean max. diam. of erythrocytes in μ	8.87	7.90	9.05	8.74	8.87	7.93	8.86	8.98
Variance of means	0.0031	0.0032	0.0180	0.0069	0.0152	0.0028	0.0036	0.0064
No. of animals	19	19	8	8	5	5	7	7
Mean corpuscular vol. of erythrocytes in μ^3	161.63	114.94	166.00	161.50	158.20	122.60	165.14	164.57
Variance of means	12.5768	9.5761	24.0714	35.0357	12.5400	6.1600	14.9085	2.7466

mates served as controls. In addition, the cardiac blood of 10 untreated adult animals was examined.

Samples of blood were obtained from the tails of the immature animals before and 2 days after each series of injections. In both immature and adult groups of animals the mean maximal diameter of the erythrocytes was determined by direct measurement and the mean corpuscular volume was calculated. The data thus obtained were analyzed statistically.[4]

Results. In the immature, developing opossum the mean maximal diameter of the erythrocytes was greater and the mean corpuscular volume larger than these respective values in adult animals of the same species (Table I). The direct injection of either normal human gastric juice or of a concentrated solution of liver extract into immature opossums significantly reduced the mean maximal diameter and the mean corpuscular volume of the red blood cells as compared with untreated litter mate controls (Table II). These changes were effected through a relative increase in the number of smaller erythrocytes. The mean maximal diameter and the mean corpuscular volume of the erythrocytes of the treated immature opossums approached but did not equal these respective values in the blood of adult opossums (Tables I and II). Normal human gastric juice and a concentrated solution of liver extract, used in the manner described in this experiment, were equally effective in reducing the mean maximal diameter and the mean corpuscular volume of the erythrocytes of opossums developing in the maternal pouch.

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[4] Fisher, R. A., *Statistical Methods for Research Workers,* 4th ed., Oliver and Boyd, London, 1932.

10969

The Local Anticonvulsive Action of Calcium Salts.*

R. BEUTNER. (With the assistance of H. Wastl and A. S. Jensen.)

From the Department of Pharmacology, Hahnemann Medical College of Philadelphia.

Using Beutner and Miley's method,[1] it was found that 200 mg per kg of procaine HCl injected intramuscularly into 56 guinea pigs caused clonic and tonic convulsions in all of the animals. The convulsions lasted 51 minutes on the average, and were preceded by hind leg stiffness. One hundred mg per kg of procaine HCl produced convulsions in 84% of 204 observations, with an averge duration of 12 minutes, while 50 mg per kg acted in only 8% of the animals with an average duration of 8 minutes.

If $CaCl_2$ was added to the procaine HCl solution and injected simultaneously, a marked decrease in the incidence of these convulsions was invariably observed. Thus, 200 mg per kg of $CaCl_2$ with 200 mg per kg of procaine HCl reduced the incidence of convulsions to 43% in 30 trials. Similarly, 100 mg per kg of $CaCl_2$ added to 100 mg per kg of the procaine solution reduced the incidence to 14% in 122 cases. This inhibiting effect of $CaCl_2$ on procaine convulsions is much less if $CaCl_2$ is injected separately from procaine.

The nature of the local action of $CaCl_2$ in reducing procaine convulsions may be clarified if one notes the effect of $MgCl_2$ added to the procaine-$CaCl_2$ mixture. Two hundred mg per kg of $MgCl_2$ was added to and injected intramuscularly in guinea pigs with 100 mg per kg of both procaine HCl and $CaCl_2$. In 137 experiments, the incidence of convulsions was 33%, or more than twice what we found it to be without $MgCl_2$. This indicates that $MgCl_2$ counteracts $CaCl_2$ locally, probably by altering membrane permeability as in the case of calcium-magnesium antagonism in the Meltzer-Auer phenomenon.

Since the locally irritating properties of $CaCl_2$ on intramuscular administration might have been a factor in the observations, we studied the effects of the less irritating organic calcium compounds by the same methods. Out of several organic calcium salts tested

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* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council of Pharmacy and Chemistry, American Medical Association.

[1] Beutner, R., and Miley, PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 279. (See also Beutner, Miller and Prussmack, *J. Pharm.*, 1936, **57**, 116.

only calcium gluconate was found to have no significant effect in inhibiting procaine or butyn convulsions. This can be correlated with the complete non-ionization of this compound, since calcium gluconate was found to be the *only* calcium salt the solutions of which are non-conductors of the electrical current. Calcium lactate, however, which is about as good a conductor as $CaCl_2$ in watery solution, appears to surpass calcium chloride, on the basis of calcium content, in power to inhibit these local anesthetic convulsions. Similarly, calcium salicylate, which is a good conductor, surpasses calcium chloride in reducing the incidence of procaine or butyn convulsions when injected simultaneously. Calcium gluconate is inefficient only when mixed and injected simultaneously with procaine. On separate injection it has some anticonvulsive action probably due to chemical decomposition.

By the same methods one may estimate the anticonvulsant effects of epinephrin HCl when given with local anesthetics. Thus, in a concentration of 1:50,000 in a procaine solution administered in a dose of 100 mg per kg, the incidence of convulsions was lowered from an expected 84% to 8%. This is, of course, a strictly local effect, as can be readily demonstrated by failure to observe a lowering of expected incidence of convulsions when relatively large doses of epinephrine HCl are injected into one leg and the procaine into the other. Ephedrine was found to be devoid of any such anticonvulsive action, even when given in relatively high doses simultaneously with procaine.

Camp[2] has called attention to the epinephrine-like action of large doses of potassium salts. When added to solutions of procaine HCl and injected with it, KCl was found to have anticonvulsant action. Thus, 100 mg per kg of KCl given with 100 mg per kg of procaine HCl reduced the expected incidence of convulsions from 84% to 59% in 48 experiments.

Tatum[3] and others have shown that the convulsant action of local anesthetics can be inhibited by various centrally acting depressant drugs, such as the barbiturates. This anticonvulsive action can, however, only be demonstrated if the barbital is administeed 15 or 30 minutes before the local anesthetic. We found that if pentobarbital, or phenobarbital, are mixed with procaine solutions and injected simultaneously, the incidence of expected convulsions is higher than if the procaine is given alone. Thus, 100 mg per kg of procaine HCl with 20 mg per kg of either barbital gave an incidence of 91% convulsions in 60 cases, instead of the expected 84% found for procaine alone. However, if these same amounts of these barbitals were injected half an hour earlier than the procaine, no convulsions appeared at all. The barbitals therefore seem to have no *local* action in inhibiting local anesthetic convulsions.

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[2] Camp, W. J. R., and Higgins, J. A., *J. Pharm.*, 1936, **57**, 376.

[3] Tatum, A. L., Atkinson, A. J., and Collins, K. M., *J. Pharm.*, 1924-5, **26**, 325; Knoefel, P. K., Herwick, R. P., and Loevenhart, A. S., *J. Pharm.*, 1930, **39**, 397.

Full details of these experiments will be published later. Meanwhile, it may be concluded that $CaCl_2$ tends to inhibit local anesthetic convulsions when administered in the same solution by altering permeability, as indicated by the fact that the addition of $MgCl_2$ to the solution counteracts this anticonvulsant action. The anticonvulsant effect of calcium salts seems to be proportional to the extent of ionization. Epinephrine and potassium ions tend to inhibit local anesthetic convulsions when administered in local anesthetic solutions by local constricting effects. Barbiturates inhibit local anesthetic convulsions *only* by central depressant action.

(The writer wishes to thank Dr. H. Wastl for her painstaking work on the animal experiments mentioned, and Mr. Arthur S. Jensen for his conductivity measurements of organic calcium salts.)

10970 P

Production of Pyrogen in Sera by Bacteria.

Co Tui and M. H. Schrift.

From the Laboratory of Experimental Surgery, New York University College of Medicine.

The occurrence of fever and chills is a relatively frequent phenomenon following intravenous serum therapy. Among the many writers calling attention to this reaction, it suffices only to mention Bullowa[1] who speaks of its high incidence in the use of refined and unrefined pneumococcus sera. The subject has been a fruitful field of research and speculation. The cause of this reaction has been variously attributed to hemolysis, to the use of anti-coagulants, to the presence of toxic fractions of proteins, and to bacterial contamination.

Felton[2] has shown the reactive principle to be in the acid precipitate of the water-insoluble protein of the sera, and intimates that it may be of bacterial origin—"for at no time has it been possible to concentrate serum without some bacterial growth." Sabin and Wallace[3] have demonstrated that anti-coagulants, Berkefeld filtration, dialysis and moderate heating have no effect in removing the reactive factors from whole blood or sera.

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[1] Bullowa, J. G. M., *Management of the Pneumonias,* Oxford Press, 1937, p. 317.

[2] Felton, L. D., and Kauffman, G., *J. Infect. Dis.*, 1931, **49**, 335.

In our previous work on pyrogen we have shown the similarity of the symptomatology following the intravenous injection of reactive infusion-fluids,[4] reactive inulin,[5] reactive acacia,[6] and the cell-free fluid of typhoid vaccine.[7] The symptomatology in the main consists of a rise in temperature, leucopenia, and in severe cases, chills and gastro-intestinal disturbances.

The present work deals with the production of pyrogen (the chill- and fever-producing factor) by inoculating normal horse sera with bacteria and incubating them for variable periods of time. Dogs were used for these experiments. It may here be mentioned that dogs were first used by Sabin and Wallace[3] in their work on the chill-producing substances in therapeutic sera. We have found dogs quite satisfactory in our work on inulin, water and acacia, and prefer them to rabbits which were used by Seibert[8] in her work on the pyrogen in distilled water. The phenomenon of leucopenia which

TABLE I.
Pneumococcal Serum—Type I (N. Y. C. Dept. of Health).*
Dog No. 34—10 kg. January 18, 1937.

Time	Temp. °F	W.B.C.†	Remarks
11:10 A.M.	101.2		
11:15	101.2	9,600	10 cc intravenously
11:45	103.6		depressed
12:00	103.6	6,500	
12:30 P.M.	103.4		shivering
1:00 P.M.	101.8		vomitus
1:30	101.8		
2:00	102.0		depressed
2:30	102.0		
3:30	101.8		
4:30	101.6		

* We are grateful to Drs. Muckenfuss and Falk of the New York City Department of Health for supplying us with material and for their interest and encouragement throughout the course of this work on pyrogen.

† W.B.C. = white-blood-cell count.

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[3] Sabin, A. R., and Wallace, G. B., *J. Exp. Med.*, March 1, 1931.

[4] Co Tui, McCloskey, Schrift and Yates, PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 297; *J. A. M. A.*, 1937, **109**, 250; *Annals Surg.*, 1937, **106**, 1089.

[5] Co Tui, Schrift, McCloskey and Yates, PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 227.

[6] Co Tui, Schrift and Ruggiero, PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 533.

[7] Co Tui, Benaglia, Ruggiero and Yates, PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 272.

[8] Seibert, F. B., *Am. J. Physiol.*, 1923, **67**, 90.

TABLE II.
Bacterial Production of Pyrogen in Normal Horse Serum.*

Organism	Incubation period	Wt of dog, kg	Vol. inj., cc	Changes in temp., °F	Changes in W.B.C., × 1000	Symptoms
Sterile control	48 hr	12	25	102.4-102.4	18. -17.3	none
Proteus vulgaris	48 ”	16	10	102.4-106.4	7.8- 0.8	shivering, vomitus, diarrhea; depressed
B. coli	48 ”	16	10	102.2-106.0	15.2- 1.9	shivering, vomitus, diarrhea
Staph. aureus	48 ”	12	25	101.4-103.4	15.8- 3.3	vomitus, shivering
Water organism “S,” genus unidentified	72 ”	12	10	100.4-102.8	14.0- 2.9	shivering
” ” “C,” ” ”	72 ”	13	10	100.8-102.2	15.0- 5.5	none
B. subtilis	72 ”	12	25	101.4-102.0	12.7- 7.0	”

* Horse serum was kindly supplied by Sharp and Dohme of Philadelphia, Pa.

accompanies this pyrogenic reaction seems to be more sensitive in dogs and affords an added check.

Table I shows the symptomatology following the injection of a known "reactive" serum. Table II represents experiments in which normal sera were inoculated with various microörganisms from culture slants, and, after incubation, and filtration through Berkefeld "W" candles, were then injected intravenously into dogs. It is significant to note that none of the sera after incubation showed perceptible turbidity, but that subcultures made from all revealed viable organisms.

It will be seen in the control experiment in Table II that the administration intravenously of 25 cc of a normal horse serum incubated at 37°C for 48 hours caused neither a change in temperature nor in blood count in the control experiment. However, the administration of from 10 to 25 cc of this same serum inoculated with bacteria gave "reactions" consisting of different degrees of pyrexia and leucopenia, the more profound cases being accompanied by vomiting and diarrhea. *Proteus vulgaris* (Exp. 2), *B. coli* (Exp. 3), *and Staphylococcus aureus* (Exp. 4) all caused severe reactions. The 2 water-organisms caused mild reactions and *B. subtilis* gave a slight or questionable reaction, with a definite leucopenia but a doubtful pyrexia. In this connection it is interesting to note that in a work to be embodied in a future report, we have found *B. subtilis* to be productive of a great deal of pyrogen in infusion-broth media.

Conclusion. The growth of some common bacteria in "normal non-reactive sera" causes them to produce a pyrogenic symptom-complex when injected intravenously into dogs. This symptom-complex consists of fever, leucopenia, and in severe cases chills and gastro-intestinal disturbances, and is indistinguishable from that produced by therapeutic reactive sera, reactive acacia, reactive inulin, or reactive infusion-fluids.

On the basis of these experiments it is suggested that the chill- and fever-producing substance in sera may be pyrogen, caused either by direct bacterial growth or by pyrogen already present in chemicals used in the purification and concentration of therapeutic sera.

10971 P

Toxicity of Acid-Soluble Typhoid Toxin for Laboratory Animals.

E. W. DENNIS.

From the Department of Bacteriology, School of Medicine, American University of Beirut, Beirut, Lebanon.

The reported data[1] indicate that the toxins characteristic of the typhoid bacillus are identified with the bacterial cell and are present in culture-filtrate only as the result of extraction or lysis of the cell. More recently Shwartzman and his colleagues have studied the "skin reactivity" of rabbits to washings and filtrates of typhoid cultures. Burnet[2] demonstrated the "Shwartzman activity" of typhoid "endotoxin" prepared by the method of Besredka. Boivin *et al.*,[3] and Topley *et al.*[4] have reported recently that certain chemically isolated components of *Eberthella typhosa* were highly toxic for rabbits and mice, and stimulated the formation of somatic "O" agglutinins when given in sublethal doses.

The present paper summarizes our observations on the toxicity of N/4 trichloracetic-acid extracts of defatted typhoid bacilli. The extracts were further purified by repeated fractional precipitation with alcohol. The purified toxin gave the following analytical data (average of 6 preparations) : N 3.1%, P 1.5%, no reducing sugar before hydrolysis, 30-32% reducing sugar (as glucose) after hydrolysis; the biuret reaction was usually negative, the Molisch reaction strongly positive, and osazones were readily formed. The toxic material appears to be a complex mixture of polysaccharides, free of native protein.

Rabbits are highly susceptible to the toxic somatic antigen-complex of *E. typhosa,* mice are less susceptible, guinea pigs are relatively resistant, while rats show little or no effects, when the toxin is injected intravenously or intra-abdominally in doses as small as 1 to 2 mg. There is, usually, a latent period of about 45 minutes. With onset of symptoms there is rapidly developing dyspnea, irregular heart rate, vascular engorgement, flaccid paralysis of the posterior extremities, and diarrhea. Death usually occurs in

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[1] For summary of the literature see Topley, W. W. C., and Wilson, G. S., *The Principles of Bacteriology and Immunity,* 2nd Edition, 1938, Edward Arnold and Co., London.

[2] Burnet, F. M., *J. Path. and Bact.,* 1931, **34**, 45.

[3] Boivin, A., and Mesrobeanu, L., *Rev. d'immunol.,* 1936, **2**, 113.

[4] Topley, W. W. C., Raistrick, H., Wilson, J., Stacey, M., Challinor, S. W., and Clark, R. O. J., *Lancet,* 1937, **1**, 252.

18-36 hours (90%), but may occur as early as 1 hour, or as late as the 5th day after injection.

Necropsy findings: Animals dying within 8 hours showed little but generalized hyperemia. In several animals that died shortly after injection, death was due to massive hemorrhage into the pericardial cavity. Microscopically there was moderate to severe capillary hemorrhage in all tissues, including the brain and spinal cord. Animals dying later than 12 hours after injection showed severe hyperemia and numerous punctate hemorrhages in all tissues; the lungs usually showed severe multiple infarction; the small intestine was filled with a bloody matrix of mucus and desquamated epithelium; the Peyer's patches were swollen, hyperemic, and showed punctate hemorrhages. Microscopically the principal features were: generalized capillary hemorrhage with consequent foci of necrosis, fragmentation of the heart muscle, and loss of follicular arrangement, due to edema, in the lymph-nodes. The adrenals showed rapid loss of chromaffin material 3 hours after injection.

The toxic somatic antigen-complex is highly active in preparing for and eliciting the Shwartzman phenomenon. There is a reciprocal relationship between the size of the preparing and eliciting doses. One of our preparations (Lot 17/A) prepared the skin of susceptible rabbits in a dose of 0.00025 mg when 5 mg of the same fraction was given intravenously 22 hours later. The reaction was neutralized by specific homologous antitoxic sera as well as by specific antibacterial antiserum. The neutralizing capacity of a serum was parallelled by its somatic "O" antibody-content. The highest doses of the toxin used for preparing the skin produced hyperemia and petechial hemorrhage at the site of injection within 18 hours.

Conclusion: The fraction containing the somatic antigen-complex of the typhoid-bacillus is highly toxic. The macroscopic changes observed are those attributed to the so-called endotoxin.[5] The histopathological changes observed indicate that the effect of the toxin is primarily on the blood vessels, damage to which results in degenerative changes in the tissues supplied. A monocytic response is not a component of the tissue-response to the toxin.

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[5] Arima, R., *Centralbl. f. Bakt.*, I Abt. Original, 1912, **63**, 424.

10972

Effect of Sulfapyridine (Dagenan) on *Brucella abortus in vitro* and *in vivo*.*

E. E. HAMANN AND I. F. HUDDLESON. (Introduced by W. L. Mallman.)

From the Department of Bacteriology, Michigan State College, East Lansing, Michigan.

In vitro. A virulent, aerobic strain of *Brucella abortus* was used in this experiment. Tryptose broth and Tryptose agar respectively were used for the *in vitro* experiment. Sulfapyridine was added to 100 cc of broth in flasks to give each of the following dilutions: 1:1,000; 1:10,000; and 1:100,000. Each dilution was made in triplicate. Three flasks of broth without sulfapyridine were left as controls. Sulfapyridine was added to the broth before autoclaving to obtain complete solution of the drug. All flasks of broth were inoculated with the same amount of dilute bacterial suspension. Petri plate counts made on each flask of broth at the beginning of the experiment averaged between 5,000 and 6,000 organisms per cubic centimeter. The inoculated broth was incubated at 37.5°C and Petri plate counts made in duplicate 10 minutes, 1, 2, 3, 5, and 7 days after addition of the organisms. The bacterial content of the inoculated broth after the various intervals of incubation was determined on dilutions of 1:100, 1:10,000 and 1:1,000,000 by placing 1 cc of each dilution in Petri plates in duplicate and adding 25 cc of Tryptose agar. The plates were incubated at 37.5°C for 96 hours and the number of colonies counted. Final counts were based on the averages of the three samples in each dilution group. The results of the final counts are shown in Table I.

TABLE I.
Number of *Brucella Colonies* per 1.0 cc of Broth After 96 Hours' Incubation at 37.5°C.

Conc. of sulfa-pyridine	Time after addition of *Brucella abortus*					
	10 min	1 day	2 days	3 days	5 days	7 days
1:1,000	5,000	36,800	64,400	34,500	1,900	230
1:10,000	5,300	163,400	123,600	103,100	3,700	250
1:100,000	5,900	180,000	320,000	2,900,000	12,300,000	28,600,000
Control	5,100	6,000,000	223,000,000	120,600,000	320,600,000	207,000,000

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* Journal Article No. 372 n.s., Michigan Agricultural Experiment Station. The writers are indebted to Merck and Company, Inc., for the Dagenan used in these experiments.

The results of this experiment show that sulfapyridine has a definite bacteriostatic action on *Brucella abortus in vitro*. The decrease in the number of oganisms is especially noticeable after 5 days in the 1:1,000 and 1:10,000 concentrations of sulfapyridine. After 7 days, there is no appreciable difference in the number of organisms in the 2 dilutions. In the 1:100,000 concentration, the decrease is less noticeable. The number of organisms in the control flasks did not vary appreciably after the second day of incubation.

In vivo. Eleven guinea pigs were used in this experiment. A newly isolated, anaerobic strain of *Brucella abortus* was suspended in sterile saline solution to a turbidity of I by the McFarland nephelometer and 0.5 cc injected subcutaneously into each guinea pig. All guinea pigs were weighed before injection. Six of the animals were divided into 3 groups of 2 each and 5 were left as controls. After an incubation period of 12 days the guinea pigs in Group I were given 6.7 mg at 11 a.m.; 6.7 mg at 1 p.m. and 3.4 mg at 3 p.m. of sulfapyridine for 10 consecutive days. Group II was treated in the same manner with the exception that the dosage was 50, 50, and 25 mg respectively. In Group III the dosage was 100, 100, and 50 mg. The sulfapyridine was suspended in water and administered orally.

The guinea pigs were re-weighed and killed 29 days after the initial dose of the drug was administered. The rapid agglutination test for brucellosis was conducted on the serum of all pigs. Duplicate cultures of the spleen, liver, and kidneys were made. All organs were examined for macroscopic lesions.

All 11 guinea pigs showed a positive reaction in a serum dilution of 1:400. *Brucella abortus* was isolated from one or more organs of all animals. Macroscopic lesions were present in the liver or spleen of all controls, and in one of the treated guinea pigs. There was a fairly uniform increase in the weight of all animals.

It is apparent that the per oral administration of sulfapyridine in large amounts over a period of 10 days had little, if any, effect on the course of *Brucella* infection.

10973 P

Intake and Loss of Radioactive Cations by Certain Marine Eggs.*

S. C. BROOKS.

From the Department of Zoology, University of California, Berkeley.

In another paper,[1] three phases of intake of radioactive alkali metal ions by Nitella[2] have been provisionally identified. Most of the intake and loss of ions by the protoplasm of Nitella appears to be in the nature of ion exchange. In the first few minutes the inorganic ions present in the protoplasm before the experiment are replaced by "tagged" ions, these ions moving at rates approximately 1×10^{-7} $GM.cm^{-2}sec^{-1}$, like that of water, rather than the previously supposed rates of 1×10^{-9} or thereabouts. This is the process termed "induced accumulation".[3] The total amount of ions corresponds approximately to the number of available salt linkages in the known volume of the protoplasm. The following loss of ions is subsequently encountered repeatedly, and it may be surmised to be due to the displacement of the tagged ions from protoplasmic salt linkages by organic ions produced in metabolism. The ions used, Na, K, and Rb, are known to modulate some phases of metabolism. The organic ions so produced appear to be replaced by tagged ions from the surrounding medium, giving "primary accumulation".[3] The presence of interrelated slow and fast processes may account for the periodic oscillation of the tagged ion content of the protoplasm found throughout at least 110 hours.

Similar experiments were done with eggs of *Urechis caupo, Pisaster ochraceus* and *Patiria miniata.* The induced accumulation of ions is shown in Table I, which also shows the calculated number of salt linkages in the same volume of protoplasm. This shows correspondence between the equivalents of cations taken up by induced accumulation and the calculated salt linkages of the protoplasm. The latter figures were obtained from the observed amounts of protein and fat in these eggs.[4] The general finding that fertilization increases the induced accumulation suggests that some salt linkages formerly masked are activated in the ensuing changes.

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* This investigation has been aided by grants from the American Philosophical Society and from the Board of Research of this University.

[1] Brooks, S. C., *J. Cell. Comp. Physiol.*, in press.

[2] Brooks, S. C., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 856.

[3] Steward, F. C., *Protoplasma*, 1935, **18**, 208.

[4] Leitch, J. L., *J. Cell. Comp. Physiol.*, 1934, **4**, 457.

TABLE I.
The Equivalents of Cations in 20 mm^3 of Eggs as Shown by the First Pronounced Maximum Attained After 15 or 30 Minutes Immersion in Solutions Containing Tagged Cations; Together with Theoretical Maximum Contents.

Radioactive ion	Species used	State of eggs	Equivalents in 20 mm^3 Observed $\times 10^7$	Calculated $\times 10^7$
Na	*Urechis caupo*	Unfertilized	18	96
,,	,, ,,	Fertilized 60 min	32	,,
K	*Pisaster ochraceus*	Unfertilized	19	137
Rb	,, ,,	,,	66	,,
,,	*Patiria miniata*	,,	62	—

The later course of cation absorption markedly shows loss phases and also primary accumulation, a reoccupation of salt linkages originally occupied by inactive cations. The rhythm of oscillations is characteristic of any given lot of eggs, regardless of the fertilization. Different lots give other rhythms, maxima usually recurring at intervals of 1-2 hours. KCN, 0.001 M, inhibits these oscillations, which therefore depend upon metabolic processes which are independent of fertilization.

It is stressed that these features of cation absorption are general for protoplasm, both plant and animal, and can be interpreted best in terms of ion exchanges, including organic ions produced possibly as so-called "waste products".

10974 P

Effect of Certain Radioactive Elements on the Metabolism of Cells.

MATILDA MOLDENHAUER BROOKS.

From the Department of Zoology, University of California, Berkeley, Cal.

In order to see whether there is any effect by the addition of radioactive elements on the metabolism of cells, radioactive Na or K was added to solutions containing *Nitella* or *Elodea* or red blood cells and the oxygen consumption was measured by the Warburg method.

For *Nitella* and *Elodea*, the solutions of NaCl and KCl were .01 M at 25°C. For red blood cells, isotonic solutions of NaCl were used, namely, 1.12% at 37°C. The solutions had activities from 2.2 to 12 mC/liter, keeping the concentrations the same. Both sheep and chicken defibrinated blood was used. CO_2 was absorbed by KOH

TABLE I.

No. of exps.	Cell	Element	pH	Activity (mC/liter)	Duration of exp. hr	Effect
2	Nitella	KCl	7.3	4	24	None
6	,,	NaCl	8.2	20	24	,,
6	,,	,,	7.3	5	4	,,
4	,,	,,	8.2	2.2	24	Slight increase
6	Elodea	,,	7.2	13		None
6	Sheep r.b.c.	,,	7.3	—	4	,,
6	Chicken r.b.c.	,,	7.3	5	4	,,

according to the usual method. The pH of the NaCl and KCl solutions was 7.2. In certain cases with pond water it was 8.2. Controls with non-radioactive salts were done in all cases. Table I gives the results.

In most cases there was no observable effect on O_2 consumption as indicated by the Warburg method. The exception is that of *Nitella* when a low activity of NaCl (2.2 mC/liter) was used, and a slight acceleration was noted. There was, however, no immediate rise in oxygen consumption within a few minutes corresponding to the rapid ion exchange as shown in the induced absorption or first phase of ion exchange as illustrated by S. C. Brooks.[1] More refined methods may show other results.

It is concluded that there is no effect upon oxygen consumption because of the radioactivity of these ions.

10975

Ultraviolet Absorption Spectra of Active and Inactive Yeast.

ERNEST W. LANDEN AND FRED M. UBER.

From the Biophysical Laboratory of the Department of Physics, University of Missouri.

The ultraviolet inactivation spectrum of yeast has been investigated by several workers, notably Wyckoff and Luyet,[1] Ehrismann and Noethling,[2] Oster,[3] and Schreiber.[4] These workers are in substantial agreement on the most effective wave-length region and on the absolute energies required to produce a given degree of inactivation. It turns out that there is a maximum effect around 2600 Å, some 500 ergs/mm^2 of incident energy being sufficient to produce 50% inactivation.

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[1] Brooks, S. C., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 856.

[1] Wyckoff, R. W. G., and Luyet, B. J., *Radiology*, 1931, **17**, 1171.

[2] Ehrismann, O., and Noethling, W., *Z. Hygiene Infektionskr.*, 1932, **113**, 597.

[3] Oster, R. H., *J. Gen. Physiol.*, 1934, **18**, 251.

[4] Schreiber, H., *Strahlentherapie*, 1934, **49**, 541.

It has been generally assumed that the effect of the radiation is on the known content of yeast nucleic acid or its component radicals. The wave-length characteristics of the inactivation curves support this assumption.[3] Nevertheless, all previous attempts to secure direct quantitative measurements of ultraviolet absorption by yeast cells were either incomplete or failures. Although an absorption curve for yeast extract has been recently published,[5] it does not necessarily correspond to the values for the intact cells. Using the method of Gates,[6] Oster[3] pressed the cells between quartz plates in order to obtain a thin layer, but without success. Lucas,[7] Köhler,[8] and Wyckoff[9] published a few photomicrographs of individual yeast cells taken with monochromatic radiation at 2750 Å but no quantitative data. The transmission curve of Ehrismann and Noethling[2] for an unmentioned species of *Saccharomyces* cells in suspension in a mixture of glycerine and water seems in serious error and obviously does not represent the absorption by the individual organisms. On the other hand, the data of Caspersson[10] on selected individual cells of a brewers' yeast by the quartz microscope method are sufficiently quantitative, save possibly for the omission of the thickness of the cells, but are for only four wave-lengths. Caspersson determined absorption as a function of age or activity of cells and found older cultures to have a lessened absorption.

The present paper reports more complete absorption data, taken at 16 wave-lengths ranging from 2300 to 3650 Å, and on both an active 2-day and an inactive 16-day culture.

The extinction coefficients for an 8-micron layer of pressed cells of *Saccharomyces ellipsoideus* were determined photoelectrically. A CsO-on-Ag photocell in a quartz envelope was employed in connection with a direct-current amplifier. Galvanometer deflections corresponding to the ultraviolet transmission through the absorbing layer or through the control layer, were read alternately.

The radiation was rendered singly monochromatic by a Hilger medium quartz spectrograph. Mercury emission lines from a small water-cooled arc furnished the source. The exit slit of the monochromator was reduced to a circular opening 0.5 mm in diameter against which the quartz slide with the sample was placed. No appreciable change in transmission by the yeast occurred during the period of the measurements at the radiation intensities used.

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[5] Loofbourow, J. R., Cook, E. S., and Stimson, M. M., *Nature*, 1938, **142**, 573.

[6] Gates, F. L., *J. Gen. Physiol.*, 1930, **14**, 31.

[7] Lucas, F. F., *J. Franklin Inst.*, 1934, **217**, 661.

[8] Köhler, A., *Z. wiss. Mikr.*, 1904, **21**, 273.

[9] Wyckoff, R. W. G., *Cold Spring Harbor Symposia*, 1934, **2**, 39.

[10] Caspersson, T., *Skand. Arch. Physiol.*, 1936, **73**, suppl. 8.

Cultures of *Saccharomyces ellipsoideus,* kindly furnished by Professor W. E. Maneval, were grown on potato-dextrose agar at a temperature of 25°C. At the end of 48 hours, 5 samples were removed for absorption measurements. The same culture was retained an additional 14 days, at which time a further series of 4 samples was taken. Hence all determinations were made on samples

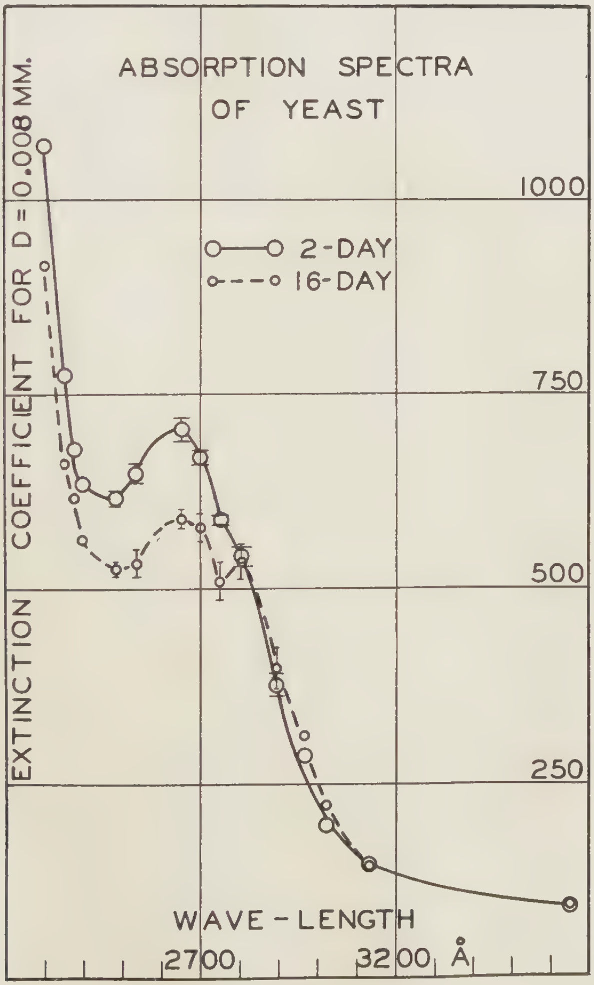

Fig. 1.

from the identical culture tube, but at a time interval of 2 weeks, in order to avoid possible variation among different cultures.

Yeast cells, obtained free from the agar substrate on a wire loop, were pressed between 2 quartz slides held apart by aluminum foil spacers. In this way a large relatively homogeneous area of uniform thickness was secured. The thickness of the layer was always 0.008 mm. A control layer of the same thickness was furnished by placing a small droplet of a 50% mixture of glycerine-water near the yeast sample on the same slide.

Results. The extinction curves determined on the 8-micron layers of the 2-day- and 16-day-old yeast cultures are shown in Fig. 1. The extinction coefficient, k, is plotted against the wave-length in Angstroms, where k is given by the expression, $k = (1/d)\log(I_0/I)$. Here I_0 is the incident flux and I the flux transmitted through the absorbing layer of thickness d in cm; decadic logarithms are used.

For the 2-day cultures, each point on the curve is an average of independent determinations on 5 samples from the same culture. Similarly, the 16-day curve represents the average of measurements on 4 different samples. Probable errors are indicated by bars and vertical lines for critical points of the curves.

The extinction coefficients for the 2-day-old yeast cultures are significantly higher than for the 16-day-old cultures in the region from 2800 Å to 2300 Å. Further, the older culture exhibits a more marked absorption band at 2800 Å.

The shape of the extinction curves obtained is similar to the inactivation curve of Oster[3] for *Saccharomyces cerevisiae* and gives some justification to Oster's assumption that the extinction curve may be derived from the inactivation curve. The quantitative agreement, however, is far less satisfactory than would appear upon casual comparison. The discrepancy is particularly noticeable for the short wave-lengths. This means presumably that other substances than the one effective in inactivation are responsible in part for the ultraviolet absorption.

Oster's[11] finding that yeast cells from a 24-hour culture are more sensitive to ultraviolet radiation than a 15-day culture would seem to be accounted for, at least in part, by the greater absorption of radiation at the earlier stage of development. This dependence of yeast cell absorption on physiological activity is in harmony with the hypothesis of Caspersson[10] regarding the increased nucleic acid metabolism of dividing cells. Caspersson's data on the extinction of selected individual yeast cells harmonize with his theory and are likewise in substantial agreement with the data presented here. His measurements having been taken at only 4 wave-lengths with the longest at 2750 Å, any increase in absorption with age at 2800 Å would not have been detected in his experiments.

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[11] Oster, R. H., *J. Gen. Physiol.*, 1934, **18**, 243.

Later data of Caspersson and Schultz[12] on the cytoplasm of *Drosophila* gut cells and on the root tips of *Allium sp.* do reveal an absorption band at 2800 Å for mature cells, while immature or actively dividing cells have their absorption maximum at 2600 Å. Characteristic of the latter type of absorption spectra are the recently obtained curves for the sporidia of *Ustilago zeae,*[13] the cytoplasm of *Drosophila* eggs,[14] and the contents of *Zea mays* pollen grains.[15]

Summary. The ultraviolet absorption spectrum of the yeast, *Saccharomyces ellipsoideus,* exhibits a maximum of 2650 Å. The extinction coefficient for the maximum decreases for inactive cells, thus revealing a secondary maximum at 2800 Å. Agreement with the theory of Caspersson on nucleic acid metabolism as a function of division rate is obtained.

10976 P

Action of Tincture of Iodine when Injected into the Pericardial Sac.

J. A. BONE AND J. W. ELAM. (Introduced by Harold C. Lueth.)

From the Department of Pharmacology and Therapeutics, University of Illinois College of Medicine, Chicago.

Since the appearance of the work of Musser and Herrmann[1] on the injection of tincture of iodine into the pericardial sac, no work has appeared on this subject. It was considered worth while to study this problem as an introduction to a general study of drugs administered by this route.

Eight healthy dogs of average size anesthetized with pentobarbital sodium were used in this series. Six dogs anesthetized with ether were used to check the effects of the anesthesia. Artificial respiration was used, blood pressure was recorded from the carotid artery, and on some animals intestinal contractions were recorded by the balloon method.

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[12] Caspersson, T., and Schultz, J., *Nature*, 1939, **143**, 602.

[13] Landen, E. W., *J. Cell. Comp. Physiol.*, 1939, **14**, 217.

[14] Caspersson, T., and Schultz, J., *Nature*, 1938, **142**, 294.

[15] Uber, F. M., *Am. J. Bot.*, 1939, **26**, in press.

[1] Musser, J. H., and Herrmann, George R., PROC. SOC. EXP. BIOL. AND MED., 1925, **23**, 212.

The heart was exposed by dividing the sternum, thus facilitating intrapericardial injections. The exposed viscera and tissues were protected with liquid petrolatum.

Usually an hour was allowed to elapse following the operative procedure before the experiment was started, to decrease the effects of shock.

Injections were made through the elevated pericardial sac, care being used not to injure the cardiac musculature. Time was permitted to allow irregularities induced by lifting the pericardial sac to be reduced to a minimum before the tincture of iodine was injected.

Following the slow injection of 2 cc of tincture of iodine (U.S.P.) there was usually a slow, gradual rise of blood pressure which lasted 5 or 10 minutes, and a concomitant increase in pulse pressure. There was also depression of intestinal activity, most probably due to the alcohol contained in the tincture. Repeated doses of tincture of iodine produced similar results.

In a second series of 4 dogs, large doses, as much as 10 cc, of tincture of iodine were injected into the pericardial sac. This amount was not fatal and results similar to those indicated above were obtained. Sixteen cc given in 2 cc doses at 15-minute intervals were not lethal. The fact that the urine gave tests for iodides indicates that the drug is absorbed from the pericardial sac.

It was found in a third series of 3 dogs that the fatal dose of tincture of iodine when injected intravenously (femoral vein) was 1 cc per kg of body weight.

A more detailed investigation is now in progress and the report will appear in the near future.

Conclusions. (1) Tincture of iodine injected into the pericardial sac is rapidly absorbed, as indicated by the presence of iodides in the urine. (2) Tincture of iodine injected in the pericardial sac effects a gradual rise in blood pressure with an increase in pulse pressure. There is also a temporary decrease in intestinal activity, which occurs after section of the vagi, and is also obtained by intravenous injection of the iodine. (3) The M.L.D. of tincture of iodine injected intravenously, when given at the rate of 0.1 cc of tincture of iodine per kg of body weight every 5 minutes, is 1 cc per kg of body weight. (4) Ten cc (1 dose), and 16 cc given in divided doses of tincture of iodine in the pericardial sac were not lethal.

10977

Irradiation of Cancer Following Injection of Colchicine.*

M. F. Guyer and P. E. Claus.

From the Department of Zoology, University of Wisconsin, Madison.

Inasmuch as cancer cells are reputed to be most vulnerable to the effects of X-rays during prophase and metaphase stages of division, and since the drug colchicine arrests mitosis in metaphase and holds it there for from 15 to 25 hours, it seemed probable that a colchicine treatment of animals bearing cancers followed some hours later by irradiation of the tumors should be a more effective treatment than X-ray treatment alone. With this thought in mind, beginning in July, 1938, the authors have experimented on 672 cancer-bearing rats including controls.

It speedily became evident that a dosage of colchicine of 0.2 mg or more per 100 g of body weight was likely to be harmful or even lethal for rats, particularly for females. A dosage of 0.1 mg per 100 g of body weight administered by subcutaneous injection, was settled upon as the standard in most of our experiments. Also, it was found that 3000 *r* of X-rays given at a single treatment were invariably lethal in from 2 to 3 weeks. While even 4500 *r* were tolerated when administered in single treatments of 1500 *r* each, if intervals of 2 weeks or more were allowed between exposures, as the experiments proceeded it became evident that smaller dosages (188 to 375 *r*) frequently repeated were more effective than larger and fewer dosages.

To determine the effects of various amounts of the drug, and particularly, the number of hours after administration that the maximal effect occurs, biopsies were made from the tumors of 5 rats at intervals of 5 hours and the number of mitotic figures in 3000 cells were counted each time in comparable fields of the same tumor. In the animal injected subcutaneously with 0.1 mg of colchicine per 100 g of body weight for example, the carcinoma showed before treatment an average of 6.3% of mitotic figures in a count of 3000 cells if one considered every cell to be in mitosis that revealed definite chromosomes. The count had risen to 15.9% at the end of 5 hours; to 20.2% at the end of 10 hours; and to 38.2% at the end of 15 hours. This was the maximum attained in this tumor. By the end of 45

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* This investigation was aided by support from the Jonathan Bowman Memorial Fund, the Wisconsin Research Foundation, the Brittingham Trust Fund, and a Research Grant from Elizabeth Hopkins Johnson.

TABLE I.
Relative Percentage of Mitosis Seen at 5-hour Intervals After Administration of Colchicine.

No. of cells counted	Hr after treatment	Percentage of mitosis following administration of amts of colchicine specified per 100 g body-wt				
		.2 mg	.15 mg	.1 mg	.05 mg	.01 mg
3000	(control) 0	5.4	6.8	6.3	9.2	6.7
3000	5	9.2	—	15.9	18.0	14.1
3000	10	20.4	21.7	20.2	22.3	13.5
3000	15	24.0	40.3	38.2	—	22.7
3000	20	51.3	31.1	19.1	41.5	17.7
3000	25	64.8	—	19.8	17.6	—
3000	30	47.8	25.6	15.6	12.3	13.8
3000	35	32.2	18.0	18.4	14.5	14.8
3000	40	—	16.0	12.3	—	11.2
3000	45	—	15.2	11.0	16.8	10.1
3000	50	8.2	15.3	—	16.9	9.0

hours the count had returned to an average of 11.0%. In biopsies of tumors from other rats (not recorded in Table I) the influence of the drug as shown by noticeably increased numbers of mitotic figures persisted in some cases beyond 72 hours.

The greater lethal effect of combined colchicine and X-ray treatment over X-ray treatment alone was most strikingly demonstrated in experiments in which cancerous tissues from rats previously given 0.2 mg of colchicine per 100 g of body weight, along with comparable carcinomatous tissues from untreated animals, were submitted to exactly the same irradiation before implantation. In our experiments both sets of tissue were removed at the same time, cut into equally thin slices and given an exposure of 1500 *r* simultaneously in the same dish. The results are set forth in Table II. They show clearly the far greater lethal effect of the combined colchicine and X-ray treatment on carcinoma than that of X-ray treatment alone or of colchicine alone. All of the 89 rats classed as "no takes" in the experiments were later implanted with untreated tumor grafts and 63 proved to be "takes" showing that these animals were not constitutionally immune to cancer transplants. The average percentage of "takes" of the Flexner-Jobling carcinoma in our Madison strain of rats is about 85 as determined from over 3000 cancer transplantations. The infrequency of "takes" of implants which have been removed from colchicine-treated rats and rayed before implantation shows, therefore, that this treatment is highly lethal for such carcinomatous tissue.

In experiments with colchicine and X-rays on living rats bearing carcinomas the results, though not so striking, still show notable advantage of combined colchicine and X-ray treatment over X-rays

TABLE II.

Number of Takes of X-rayed (1500 *r*) Carcinoma Implants from Colchicine-treated Rats (0.2 mg per 100 g of body-weight) and of Similarly Rayed Carcinoma Implants from Rats Not Treated with Colchicine.

	Colchicine and X-ray	X-ray only	Colchicine only
Series XVI			
No. of rats used, 48	16	16	16
Results	Takes 2 No takes 14	Takes 15 No takes 1	Takes 14 No takes 2
Series XIX			
No. of rats used, 48	16	16	16
Results	Takes 6 No takes 10	Takes 10 No takes 6	Takes 11 No takes 5
Series XXX			
No. of rats used, 105	53	52	0
Results	Takes 9 No takes 44	Takes 46 No takes 6	

alone or colchicine alone, when moderate and frequent treatments with both X-rays and colchicine are employed.

It was not until some 20 series of experiments involving several hundred rats had been made that a significant difference was observed. In the earlier experiments even after the maximal toleration of X-ray and of colchicine dosage had been determined, there was heavy mortality among the treated animals and little to choose between the retardative or curative effects of combined X-rays and colchicine and those of X-rays alone as long as the irradiation dosage was as high as 1500 *r* or even 750 *r* a treatment for the 3 or 4 treatments given at intervals of about 2 weeks. Each kind of treatment, including colchicine alone, showed inhibitive effects and even cures but the differential benefits did not appear significantly between: (1) the combination of colchicine and X-rays; and (2) irradiation alone, until the rayings were reduced to 375 *r* a treatment and given at intervals of a week. The differences were still more marked when the irradiation was reduced to 188 *r* and given twice a week notwithstanding the increased hazards of such frequent anesthetization to the tumor-bearing rats.

Figure 1, for example, represents a graph based on our series XXIX, numbering 91 rats (32 receiving colchicine and X-ray treatment; 29 receiving X-rays only; and 30 receiving colchicine only). Treatment was begun 16 days after implantation when the tumors had reached a size of from 1 to 1.5 cm and were all actively growing. The colchicine dosage was 0.1 mg per 100 g of body weight given each time 15 hours before irradiation; rayings of 188 *r* were given each irradiated animal twice weekly. The perpendicular line represents size in centimeters, the horizontal, time in week intervals to

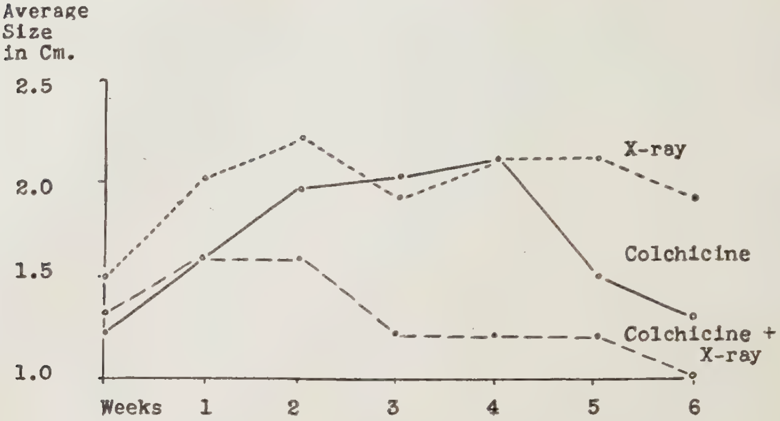

FIG. 1.

the end of the sixth week. Each week the tumors of all the surviving animals were measured (length by breadth) and the average for each group was recorded. From the graph it is evident that the tumors submitted to colchicine and X-ray treatment grew notably less on the average than those of either of the other classes. The graph for "colchicine only" is invalidated somewhat by the fact that 10 of the rats died one hot night, so that the later measurements were averaged on a smaller number of surviving individuals in this group. At the end of the sixth week 15 had been completely healed with 4 dead in the colchicine and X-ray group; 11 had been healed with 3 dead in the X-ray only group; and in the colchicine only group 8 were healed and 10 were dead.

Summary: X-rays were markedly more lethal to bits of carcinoma from colchicine-treated animals than to similar pieces of carcinoma from untreated animals, as shown by the infrequency of "takes" when implanted in susceptible animals. By administering colchicine to rats bearing actively growing carcinoma 15 to 18 hours before irradiation the effectiveness of X-rays in completely destroying or in retarding the growth of the cancer was increased. Light, frequent dosages of X-rays were less injurious to the rats and more effective in securing disappearance of the tumors than heavy, infrequent dosage.

10978

Preparation of Stable Fibrinogen for Study of Streptococcal Fibrinolysin.

L. R. CHRISTENSEN AND L. R. JONES.

From the Department of Bacteriology, St. Louis University School of Medicine, St. Louis, Mo.

In studies on streptococcal fibrinolysin it would be distinctly advantageous to have available a stable fibrinogen which could be maintained under storage conditions for a reasonable period.

Recently, Pomales-Lebron and Morales-Otero[1] have reported a technique for preserving whole plasma by a method involving freezing and desiccation in a high vacuum. Such preserved plasma, although shown to be suitable for fibrinolysis studies over a period of 8 months, must be secured from donors whose plasma is known to be susceptible to fibrinolysis. An obvious disadvantage of the method is that donors must be selected on the basis of a satisfactory preliminary test. Furthermore, we have observed that plasma clots much more slowly and undergoes lysis more slowly than does isolated fibrinogen.

Since the isolated fibrinogen fraction apparently does not carry the resistance factor which is present in many individuals, it is evident that pooled plasma from several individuals would constitute a satisfactory and convenient source of material if a method were available for preserving the isolated fibrinogen. Ferguson and Erickson,[2] in studies on blood coagulation, have preserved a highly purified fibrinogen, prepared from dog plasma, by a rather elaborate technic, involving prefreezing and subsequent desiccation in special apparatus. Fibrinolysis studies have not been reported with their product.

This report embraces the details of a simple method for isolating and preserving fibrinogen in such a state that it can be stored for considerable periods of time, maintaining all the while the ability to redissolve, and upon addition of thrombin, to yield a clot suitable for the determination of fibrinolysin in streptococcus cultures. The method is as follows: 1. Pooled plasma is diluted with an equal volume of distilled water, and filtered through a Seitz or Berkefeld filter. 2. The fibrinogen is precipitated by: a. Addition of one-fourth volume of saturated ammonium sulfate, or b. Addition of one and one-half volumes of saturated sodium chloride solution. 3. The resultant precipitate is separated by gentle centrifugation and placed in a Pyrex vacuum desiccator over phosphorus pentoxide. 4. The desiccator is then evacuated for one to 2 hours with a Cenco Hyvac pump. At the end of this time the stopcock is closed and the material allowed to remain in the desiccator overnight. 5. Upon removal, the dry material is easily broken up with a spatula or in a mortar. This dry material has been maintained at refrigerator temperature in a stoppered container for several months without evident deterioration.

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[1] Pomales-Lebron, A., and Morales-Otero, P., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 609.

[2] Ferguson, J. H., and Erickson, B. N., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 425.

In the above method, filtration serves to remove platelets and other solid material. In our experiments, satisfactory results have followed the use of either ammonium sulfate or sodium chloride as a precipitant. One precipitation with either apparently causes little or no denaturation of the fibrinogen. However, in the case of one lot of fibrinogen which was reprecipitated 6 times and then dried, the material, when redissolved, failed to give a satisfactory clot, indicating more extensive denaturation.

In this vacuum-desiccation, degassing and "snap-freezing" are accomplished in 5-10 minutes. The material has a porous and brittle consistency, (which probably facilitates resolution) and can be dissolved readily in M/100 phosphate buffer (pH 7.4) to yield a solution of the desired concentration.

Pooled, oxalated plasma, when stored for as long as 2 weeks before precipitation, often yields a product in which there is apparently some denaturation of the fibrinogen, since the dried material is somewhat less soluble and the resultant clot somewhat less firm. To compensate for this deficiency, it may be necessary to use more than the calculated amount of the dried material in order to make a satisfactory fibrinogen solution. In occasional samples we have noted the presence of some insoluble material which, while it does not interfere with fibrinolysin studies, may easily be removed by filtration if desired.

The dissolved fibrinogen clots readily (in a few seconds) upon addition of rabbit or human thrombin prepared according to the method of Tillett and Garner.[3] The clot is a stable one and does not undergo spontaneous lysis.

Tests were made with 40 strains of Group A streptococci, 2 of "Human" C, and 4 of Group G, using the technique of Tillett and Garner.[3] This technique involves the clotting of a mixture of diluted fibrinogen solution and streptococcus culture with thrombin, and the observation of subsequent lysis. Only one strain (Group A) failed to lyse fibrin clots from fibrinogen prepared in the above manner. This strain was also negative with a plasma clot. In tests made with known non-lysing strains of streptococci, the clots showed no sign of lysis after remaining overnight in the water bath. All other groups of streptococci tested, as well as several unidentified species of bacteria, failed to lyse the fibrin clot.

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[3] Tillett, W., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

Preliminary experiments indicate that this fibrinogen preparation will also serve for the determination of staphylococcus coagulase.

10979

Effect of Adrenalectomy on the Growth of Mammary Glands in Underfed Albino Rats.*

Earl O. Butcher.

From the Biological Laboratory, Hamilton College, Clinton, N. Y.

It has been found[1] that growth of hair is greatly accelerated by bilateral adrenalectomy in underfed rats. Since the mammary glands are similar in origin and location to the hair bulbs, the present experiments have been made to see if these glands, likewise, grow more rapidly after adrenalectomy in underfed animals.

After weaning at the age of 22 days, rats were isolated and thereafter fed 2 g of ground Purina dog chow each morning and 2 each evening. Rats gained very little weight on this quantity of food. Females were adrenalectomized via the dorsal approach usually when about 30 days old while unoperated litter mates served as controls. Three per cent NaCl solution was kept accessible for the adrenalectomized animals.

Usually 10 to 15 days after adrenalectomy, the adrenalectomized rats and their unoperated and underfed litter mate controls were killed and the skins of their ventral body walls were fixed in Bouin's fluid. The mammary glands were then dissected out, stained, cleared and mounted.

When the mammary glands (Figs. 1 and 2) of the adrenalectomized and control animals are compared, many more bud-like projec-

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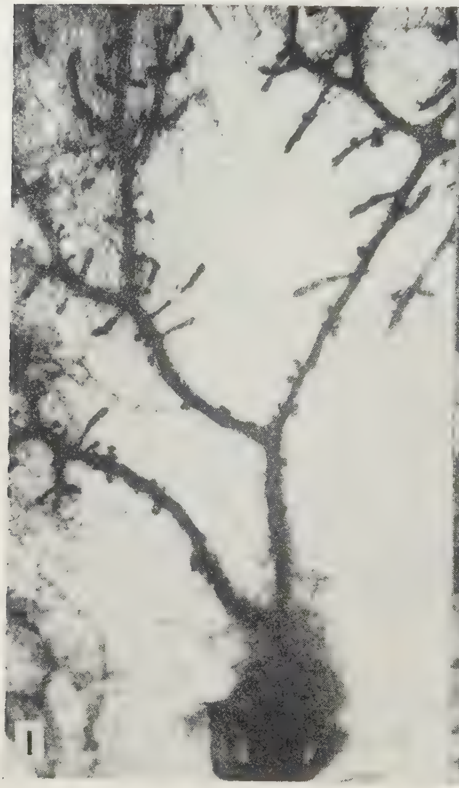
* I am indebted to Mr. Hobart Parkhurst for technical assistance in these experiments.

[1] Butcher, E. O., *Endocrinology*, 1939, **25**, 787.



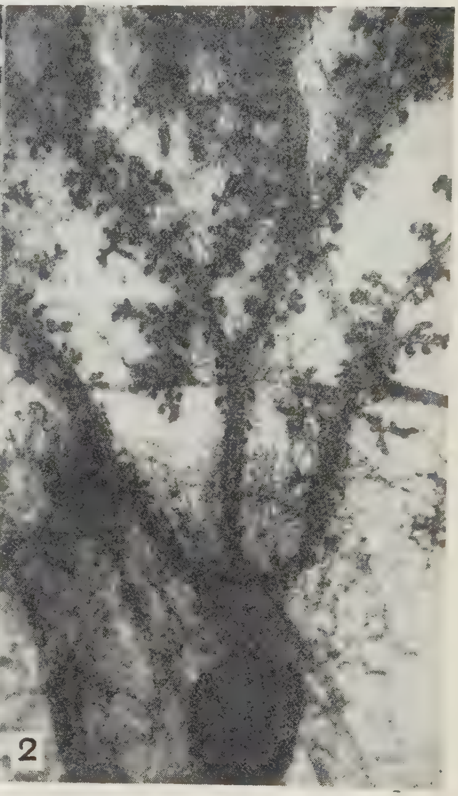


FIG. 1.

Whole mount of third thoracic mammary gland of a 40-day-old rat, underfed since the age of 22 days (body weight, 27th day—43 g, 40th day—44 g, diameter of gland—6 mm). × 17.

FIG. 2.

Whole mount of third thoracic mammary gland of a litter mate 40-day-old rat, underfed since the age of 22 days, and adrenalectomized when 27 days old (body weight, 27th day—41 g, 40th day—47 g, diameter of gland—9 mm). ×17.

tions are seen along the course and at the ends of the ducts in the adrenalectomized rat. The gland of the adrenalectomized animal also covers a larger area than the gland of the control animal.

Since increased sexual activity is said to follow adrenalectomy in male rats,[2] there existed the remote possibility that the ovaries might become more active after adrenalectomy and induce the more rapid growth of the mammary glands. Accordingly, 2 underfed rats of a litter were both adrenalectomized and ovariectomized, 2 were adrenalectomized, while the 2 or 3 others of the litter were unoperated. All the animals of the litter were sacrificed about 10 days after the operations. The mammary glands of the adrenalectomized and ovariectomized animals were comparable to the glands of adrenalectomized rats, and possessed more bud-like projections than the glands of the unoperated rats. These results were confirmed by the use of other litters. It was thus evident that the ovary was not responsible for the changes in the mammary glands of the adrenalectomized underfed individual. It seems quite probable that adrenalectomy allows more substances which are necessary for the growth of the mammary glands to pass through the capillary walls into the tissue spaces.

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[2] Marine, D., *Glandular Physiology and Therapy*, Am. Med. Assn., 1935, 331.

Summary. The mammary glands of underfed albino rats grow more rapidly upon adrenalectomy. This enlargement is not due to increased sexual activity resulting from the adrenalectomy.

10980

Relation of Skim Milk Feeding to Cataract Production.*

C. F. Krewson, E. J. Schantz, and C. A. Elvehjem. (Introduced by E. B. Hart.)

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wis.

Mitchell and Dodge[1] reported that 68% of the rats placed on a synthetic diet containing 70% lactose developed mature bilateral cataracts and on a diet containing 50% lactose, 27% of the rats became similarly afflicted. Since skim milk solids contain about 50% of lactose, it was thought that skim milk might be dangerously high in lactose. However, cataracts have never been observed in this laboratory in rats on a skim milk or whole milk diet but have been observed in rats receiving skim milk containing added galactose. In this connection it was thought advisable to make a study of the relation of lactose and galactose to nutritional cataract in animals on a skim milk diet.

Day[2] and others[3] have reported a type of nutritional cataract

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were aided in part by grants from the Wisconsin Alumni Research Foundation and the Works Progress Administration.

[1] Mitchell, H. S., and Dodge, W. M., Jr., *J. Nutrition*, 1935, **9**, 37.

[2] Day, P. L., Darby, W. J., and Langston, W. C., *J. Nutrition*, 1937, **13**, 389.

[3] O'Brien, C. S., *Arch. Ophth.*, 1932, **8**, 880; Yudkin, A. M., *J. Am. Med. Assn.*, 1933, **101**, 921; Guha, B. C., *Nature*, 1935, **135**, 395; Bourne, M. C., and Pyke, M. A., *Biochem. J.*, 1935, **29**, 1856.

caused by riboflavin deficiency and it has been found that this type of cataract is different than that caused by feeding lactose and galactose.

Experimental Skim Milk Diets. Because young rats could not be raised on mineralized skim milk alone or on rations high in galactose, it was necessary to raise them on mineralized whole milk until they weighed 80 to 100 g each and then change them to the experimental diets of fresh mineralized skim milk containing the dissolved sugars. All animals were irradiated 10 minutes daily and 10 micrograms of crystalline carotene were added to each 100 cc of milk. The onset of cataract was determined by the first appearance of opacity in the lens of the eye. No instruments were used to identify any other changes in the eye structure.

Observations were made on 40 rats on a skim milk diet for 125 to 150 days and no indication of cataract was evident. After these observations, rats were placed on skim milk with added lactose and galactose. Table I shows a summary of the experimental results obtained on skim milk diets. Skim milk with sufficient galactose added to make the free galactose content of the solids 20% did not cause cataract within 90 days. However, when the solids were made to 30% galactose, bilateral cataracts developed in all animals in 28 to 30 days. When the galactose content of the solids was made to 40% cataracts developed in 20 to 23 days. Addition of lactose to skim milk to make the lactose content of the solids 70% did not cause cataract within 90 days.

In view of Day's work,[2] additional quantities of riboflavin were fed with the skim milk with added galactose to determine if there existed any relation between the riboflavin content of milk and the incidence of cataract in these experiments. However, feeding 100

TABLE I.
Skim Milk Diets and Results Obtained with Each Diet.

Diet Skim Milk*	No. of rats	Cataract
	4	None within 150 days
Plus lactose to make lactose content of solids 70%	3	” ” 90 ”
Plus equal amounts of glucose and galactose to make sugar content 70%	3	” ” 90 ”
Plus galactose to make 20% of solids	3	” ” 90 ”
Plus galactose to make 30% of solids	3	All animals, both eyes, 28-30 days
Plus galactose to make 30% of solids plus 100 gamma riboflavin per rat per day	3	” ” ” ” 28-30 ”
Plus galactose to make 40% of solids	3	” ” ” ” 20-23 ”
Plus galactose to make 40% of solids plus 100 gamma riboflavin per rat per day	3	” ” ” ” 20-23 ”

* Previous observations made on 40 rats not included in this table.

micrograms of riboflavin per rat per day did not delay the onset of cataract in any cases.

Synthetic Diets. In connection with this work, synthetic lactose and galactose rations were fed. The ration had the following composition:

Sugar (see Table II)	70
Purified casein (ether extracted)	20
Brewers yeast (ether extracted)	6
Salts	4

One microgram of crystalline carotene was added to each gram of ration and the animals were irradiated 10 minutes each day. Since it has been shown[4] that the blood sugar of animals on skim milk is higher after feeding than of animals on whole milk, the above ration was made fat-free and lard added in some experiments to determine if fat had any effect on cataract formation. The data obtained (Table II) are quite similar to those obtained on the skim milk diets. Of the 12 animals fed the basal diet containing 70% lactose only one showed a slight sign of cataract. When the basal ration contained 35% of galactose and 35% of dextrin or glucose bilateral cataract appeared in all animals in 28-30 days. On the basal ration containing 60% galactose and 10% dextrin bilateral cataract appeared in all rats in 17 to 20 days. Addition of 20% of fat to the ration containing 60% of galactose apparently had no effect other than dilution of the ration since cataracts appeared in 18 to 22 days. Addition of fat and skim milk to the lactose rations as shown in Table II was of no value since cataracts did not appear on the 70% lactose ration alone.

It is apparent from these data that the development of cataract under the conditions of the experiment described is dependent upon

TABLE II.

Synthetic Rations and Results Obtained with Each Diet.

Basal Ration	No. of rats	Cataract
(70% lactose)	4	One in 60 days, none in others within 90 days
(70% lactose) plus 15 cc skim milk per rat per day	4	None within 90 days
(70% lactose) 80 parts, lard 20 parts	4	” ” 90 ”
(galactose 35%, dextrin 35%)	4	All animals, both eyes, 28-30 days
(galactose 60%, dextrin 19%)	4	” ” ” ” 18-20 ”
(galactose 60%, dextrin 10%) 80 parts and lard 20 parts	4	” ” ” ” 18-22 ”
(70% lactose) (later experiment)	8	None within 90 days

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[4] Schantz, E. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1938, **122**, 381.

the amount of galactose in the diet. It is also apparent that the rats used in this work were much less susceptible to this type of nutritional cataract than the animals used by Mitchell, since cataracts could not be produced on a 70% lactose diet. Mitchell[5] has shown that different strains of rats vary in their susceptibility to cataract and it is possible that rats very susceptible to cataracts would develop this condition on a skim milk diet.

No cataracts developed in rats on a 70% lactose ration, with the exception of one slight case in one animal, while all animals developed definite cataracts in 20 to 30 days on a ration containing 35% galactose and 35% dextrin. It would appear rational to explain the above observations on the premise that during the hydrolysis of lactose in the intestinal tract galactose is liberated slowly enough for the animal to metabolize this sugar, whereas, when free galactose is ingested the system is then flooded with galactose and cataract results. Mitchell[6] has shown cataract formations of this variety to be paralleled with high blood galactose. Since the onset of cataract was not delayed by the addition of riboflavin to the milk diets, the cataracts produced by the feeding of galactose are undoubtedly different than those resulting from riboflavin deficiency. The fat added to the ration apparently acted as a diluent in the ration. However, the presence of fat does slow up the absorption of, and probably aids, in the metabolism of galactose, which may account for some of the delay.

Conclusions. The development of cataract in the rat is dependent upon the amount of galactose consumed in the diet. Skim milk alone was not sufficiently high in lactose to cause cataract formation in the rats used in this work.

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[5] Mitchell, H. S., Merriam, O. A., and Cook, G. M., *J. Nutrition,* 1937, **13**, 501.

[6] Mitchell, H. S., *J. Nutrition,* 1936, **12**, 447.

10981

Further Studies on Relation of Fat to Utilization of Lactose in Milk.*

E. J. SCHANTZ AND C. F. KREWSON. (Introduced by E. B. Hart.)

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wis.

It was found in our laboratory[1] that rats placed on a whole milk diet mineralized with iron, copper, and manganese made very efficient use of the milk sugar but when placed on a mineralized skim milk diet 20-30% of the ingested galactose was lost in the urine. Natural fats such as lard, corn oil, coconut oil, and linseed oil could be substituted for butter fat in the milk without loss of galactose. Synthetic triglycerides such as palmitin and olein were effective while caproin was not effective in preventing the loss of galactose in the urine.

In view of these results it was thought advisable to test the effectiveness of several other triglycerides including odd chain fatty acids. It was also considered advisable to feed salts of the lower fatty acids and high levels of glucose along with skim milk.

Experimental Effect of Even Chain Triglycerides. The triglycerides of caproic, caprylic, capric, and lauric acids were synthesized from the crude acids obtained from Eastman Kodak Company. These glycerides were homogenized into fresh skim milk at a level of 4%. The metabolism experiments were performed on rats weighing between 100 and 140 g and which had been raised on mineralized whole milk. The animals were placed on a skim milk diet until they began to lose considerable galactose in the urine, which required 10-12 days. At this time 24 hour metabolism periods were run on the rats receiving the synthetic triglycerides homogenized into the skim milk. The results obtained show that caproin, caprylin, and caprin were not effective within a 10-day period in preventing any appreciable loss of galactose but laurin was quite effective. These same animals stopped losing galactose in the urine within 3 or 4 days when corn oil was substituted in the skim milk. The 18 carbon keto acid, licanic, was also fed and found to be effective.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were aided in part by grants from the Wisconsin Alumni Research Foundation and the Works Progress Administration.

[1] Schantz, E. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1938, **122**, 381.

Effect of Feeding Odd Chain Acids. The glyceride of margaric acid, which had been isolated from natural sources[†] was fed in skim milk, and it was found to be quite effective in preventing the loss of galactose. Since Schriner, Fulton, and Burks[2] have pointed out that an equimolecular mixture of palmitic and stearic may be mistaken for margaric acid and that a mixed melting point is not conclusive evidence for identification, it was considered advisable to feed a synthetic odd chain acid. Pentadecylic acid was synthesized[3] and the triglyceride made and fed with skim milk. A slight response was obtained in some cases but no definite and lasting effect was noticed in preventing the loss of galactose in the urine.

Effect of Various Salts. Since some difficulty was encountered in feeding the lower triglycerides due to liberation of free acid in the milk, the sodium salts of caproic, caprylic, and capric acids were fed at levels of 4% in skim milk. These salts did not stop the loss of galactose in the urine but in some cases tended to increase the excretion of galactose. Feeding 2 per cent of NaCl or Na_2HPO_4 in the skim milk increased the excretion of galactose as much as 20%. The action of KCl was similar but not as pronounced as NaCl or Na_2HPO_4. The feeding of these salts with whole milk caused some animals to lose small amounts of sugar in the urine.

Effect of Feeding High Levels of Glucose. Previously it was reported[1] that feeding glucose along with the skim milk prevented some loss of galactose in the urine of some animals. Later it was

TABLE I.
Effect of Various Fatty Acids, Salts, and Glucose on Galactose Metabolism.

Substance	Level Fed, %	Effectiveness
Caproin	4	Very slight, if any
Caprylin	4	” ” ” ”
Caprin	4	” ” ” ”
Lauric acid	4	Good response
Licanic acid	4	” ”
Margarin	4	” ”
Pentadecylin	4	Poor response
Na salt of caproic acid	4	” ”
Na ” of caprylic acid	4	” ”
Na ” of capric acid	4	” ”
NaCl	2	Increased excretion of galactose
Na_2HPO_4	2	” ” ” ”
KCl	2	” slightly in some cases
Glucose	8	Very slight
”	10	” ”

[†] Obtained as a gift from R. W. Clark, Pharmacy Department, University of Wisconsin.

[2] Schriner, R., Fulton, J., and Burks, D., Jr., *J. Am. Chem. Soc.*, 1933, **55**, 1194.

[3] Krewson, C. F., Doctorate Thesis, University of Wisconsin, now being compiled.

deemed advisable to replace the calorific value of the fat with glucose, which would require feeding 8% of glucose. Glucose was fed at levels of 8 and 10% in the skim milk and the galactose excretion decreased 10 to 30%. The sugar excreted in the urine on high levels of glucose was non-fermentable.

A summary of the results obtained is shown in Table I.

It is apparent from these experiments that the fatty acids of 12 carbon atoms or more are the most effective in preventing the loss of galactose in the urine. No explanation can be offered until more is known about how the fatty acids function in galactose metabolism.

The contradiction between the results obtained with margaric acid and pentadecylic acid may be explained by the fact that the margaric acid which was isolated from natural sources may have been a eutecic mixture of even chain acids.

The fact that Na_2HPO_4, and NaCl increased the excretion of galactose is surprising in view of the fact that McQuarrie, Tompson, and Anderson[4] found that sodium salts caused a decrease in glycosuria in diabetic children. Crabtree and Longwell[5] found that rats on a high salt diet containing glucose deposited almost twice as much liver glycogen as rats on a low NaCl diet. This phenomenon has not been further investigated by us, but it points again to the difference between the metabolism of galactose and glucose.

Feeding high levels of glucose in skim milk to replace the calorific value of the fat apparently does not take the place of fat. It is very likely that the small decrease in the excretion of galactose when glucose was fed was due to the retarding action of glucose upon the absorption of galactose.

Conclusions. Even chain fatty acids containing 12 carbon atoms or more when fed with skim milk to the extent of 3 to 4% were effective in preventing the loss of galactose in the urine while acids with less than 12 carbon atoms were not effective in preventing this loss. An odd chain fatty acid (pentodecylic) was not effective in preventing loss of galactose. Feeding glucose at levels of 8 and 10% in skim milk did not prevent the loss of galactose.

The excretion of galactose was increased as much as 20% by feeding 2% of NaCl or Na_2HPO_4 in the skim milk. KCl was not as effective in increasing the excretion of galactose as NaCl or Na_2HPO_4. These salts caused some excretion of galactose on whole milk diets.

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[4] McQuarrie, I., Tompson, W. H., and Anderson, J. A., *J. Nutrition*, 1935, **11**, 77.

[5] Crabtree, D. G., and Longwell, B. B., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 705.

10982

Inhibition by Cortin of the Blood Sugar Changes Caused by Adrenalin and Insulin.

Hans Selye and Christiane Dosne.

From the Department of Anatomy, McGill University, Montreal, Canada.

It has been shown by previous investigators that cortin and crystalline cortin-like compounds when given in excess to normal animals have no hyperglycemic or diabetogenic action,[1] while in the partially pancreatectomized animal, these substances are strongly diabetogenic.[2, 3] These observations have been considered to prove that normally, insulin produced by the animal's own pancreas inhibits the inherent sugar-mobilizing action of cortin, a mechanism which is disturbed after partial pancreatectomy. The action of crude cortical extracts on adrenalin hyperglycemia has been examined in the rabbit by Oda[4] who claimed that such extracts may inhibit adrenalin hyperglycemia. On the other hand, in the cat Britton and Silvette[5] and in the rabbit Sabatucci[6] obtained negative results. Oda[4] claimed, furthermore, that cortical extracts increase the hypoglycemic effect of insulin, an observation which would appear to be in contradiction with the above mentioned findings on the partially pancreatectomized animals. The following experiments have been performed to clarify these questions.

In all experiments reported in this communication, adult "hooded" male rats weighing between 160 and 249 g have been used. The animals were distributed in such a manner as to make the average weight of each experimental group approximately 200 g. The rats were fasted during the 24 hours preceding the beginning of the experiment. In our first experiment, 6 rats received a subcutaneous injection of 0.2 cc of a 1:1000 solution of adrenalin. They were sacrificed for blood sugar determination (Shaffer, Hartmann and Somogyi's method) 30 minutes later. Another group of 6 rats received 1 cc of Wilson's cortical extract*) 15 minutes prior to the injection of the same amount of adrenalin as was used in the first group. 0.03 cc of this cortical extract contained 1 unit of cortin as defined by Selye and Schenker.[7] The average blood sugar in the group receiving adrenalin alone was *191* mg % with variations between 170 and *228* mg % while in the group pretreated with cortin, the average blood sugar was *145* mg % and the extreme variations, 126 to 170 mg %. We repeated this experiment on another group of rats under identical conditions except that the animals were killed 1 hour after the adrenalin injection. In this experiment, the group receiving adrenalin alone had an average blood sugar of *240* (range: 224-256), while that pretreated with cortin had an average blood sugar of *206* mg % (range:178-219).

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[1] Grollman, Arthur, *Am. J. Physiol.*, 1938, **122**, 460.

[2] Kendall, E. C., *XVI Internat. Physiol. Cong.*, Zurich, August, 1938.

[3] Long, C. N. H., Fry, E. G., and Thompson, K. W., *Proc. Am. Physiol. Soc.*, Baltimore, 1938.

[4] Oda, Y., *Fol. endocrin. jap.*, 1928, **4**, 53.

[5] Britton, S. W., and Silvette, Herbert, *Science* (N.Y.), 1931, **1**, 373.

[6] Sabatucci, Nicoletta, *Boll. Soc. ital. Biol. sper.*, 1934, **9**, 655.

* We are greatly indebted to Dr. David Klein of the Wilson Laboratories for supplying us with the cortical extract.

These experiments showed that the hyperglycemic response to adrenalin is decreased in animals pretreated with cortin but we felt that blood sugar curves would be necessary to determine whether the inhibition is real or merely apparent and due to a delay of the maximum response. For this purpose, we repeated the experiment on another group of 12 rats, 6 of them receiving adrenalin alone, and 6 in combination with cortin in the same manner as in the previous experiments. Blood was taken at intervals from the heart for sugar determinations. Table I summarizes our results which indicate that the inhibition was real, inasmuch as the blood sugar in the group receiving cortin was not only inhibited at a certain time but actually never reached as high a level as after adrenalin alone. Since the maximum blood sugar level is not necessarily attained at the same time in each case, the peak values have been italicized in the tables and it was found that the average of all these peak values is 184 in the case of the first group as compared with 151 in the second group which received both hormone preparations.

In order to establish how adrenalin influences insulin hypoglycemia, we performed another experiment on 12 fasted rats giving *2* units of Toronto insulin subcutaneously to all animals. This injection was preceded in 6 rats by the subcutaneous administration of 1 cc of cortin, 15 minutes before the insulin. Since these animals were fasted for 20 hours only, the initial blood sugar was not as low as it usually is at the beginning of these experiments. These results have been summarized in Table II from which it is evident that the hypoglycemia produced by insulin has also been inhibited by cortin to some extent. The average of the italicized numbers, which represent the maximum hypoglycemia of each animal, is *39*

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[7] Selye, Hans, and Schenker, Victor, PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 518.

TABLE I.
Effect of Cortin on Adrenalin Hyperglycemia.

No. of animals	1	2	3	4	5	6	Avg
			Adrenalin.				
Fasting blood sugar	96	87	96	79	96	71	88
Time after adrenalin injected							
0 hr 30 min	145	136	143	136	145	132	140
1 " 30 "	174	170	163	*182*	166	*178*	*172*
2 " 30 "	*211*	*198*	*163*	178	*170*	170	183
7 "	96	92	104	111	119	79	100
			Adrenalin + Cortin.				
Fasting blood sugar	79	87	100	75	96	96	90
Time after adrenalin + cortin injected							
0 hr 30 min	79	87	104	107	100	119	99
1 " 30 "	119	124	145	128	128	*141*	*130*
2 " 30 "	*128*	*145*	*186*	*170*	*136*	sample lost	153
7 "	87	92	128	96	75	79	89

in the case of treatment with insulin alone and *60* in the case of insulin and cortin. A repetition of this experiment on rats fasted for 24 hours and receiving only 1 unit of insulin gives similar results. Thus 2 hours after the insulin injection, the average blood sugar was *38* mg % (range: 23-58) while in the group receiving insulin and cortin, it was *60* mg % (range: 54-67). The average of the maximum hypoglycemia values was *28* in the former and *42* in the latter group. The fact that these values are lower than in the group summarized in Table II is due to the longer fasting period which led to a lower initial blood sugar value (average 82 mg %) in this experiment. In both groups, however, the inhibition of the hypoglycemic effect of insulin appears to be quite obvious. The fact that cortin in the dosage employed has no significant effect on the blood

TABLE II.
Effect of Cortin on Insulin Hypoglycemia.

No. of animals	1	2	3	4	5	6	Avg
			Insulin.				
Fasting blood sugar	111	107	100	92	111	100	103
Time after insulin injected							
1 hr	67	*54*	43	*39*	63	33	49
2 "	*31*	58	*31*	43	*47*	*31*	*40*
4 "	54	63	71	87	71	87	72
6 "	79	92	71	87	75	87	82
			Insulin + Cortin.				
Fasting blood sugar	107	92	111	103	107	96	102
Time after insulin + cortin injected							
1 hr	75	67	75	96	87	*43*	74
2 "	*71*	*54*	67	*67*	*67*	71	*66*
4 "	92	71	*58*	75	92	71	76
6 "	96	79	71	87	79	83	82

sugar by itself, was shown in another group of 6 rats in whom the blood sugar after 24 hours fasting was 89 mg % on the average (range: 83-92). One hour after subcutaneous injection of 1 cc of cortin, it was 89 mg % (range: 87-92) and one hour after that 92 mg % (range: 76-105).

Summary. Experiments on the fasted rat indicate that both the hyperglycemia caused by adrenalin and the hypoglycemia following insulin administration may be inhibited, though not completely suppressed, by cortin. It appears that cortin exerts a stabilizing effect on the blood sugar not unlike that obtainable by certain pituitary extracts as described by Neufeld and Collip.[8]

10983

Effect of Methylcholanthrene on Latent Period of Breast Tumors in Dilute Brown Mice.*

G. BURROUGHS MIDER† AND JOHN J. MORTON.

From the Department of Surgery, the University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The production of breast tumors in non-susceptible female mice of strains IF, CBA, JK, and NH by subcutaneous injection of methylcholanthrene was reported almost simultaneously in England[1] and in this country.[2] Observations in this laboratory also indicate that methylcholanthrene may affect the production of mammary carcinoma in a strain of mice that is known to develop the tumors spontaneously.

Breeding female dilute brown (Little dba) mice have a high incidence of spontaneous mammary carcinoma.[3] Subline 212, on which our experiments were performed, has a lower incidence of breast tumors than other members of the strain. Forty-two identified breeding female mice of this line have been observed for at least one year. Fourteen of them developed one or more spontaneous breast tumors when 250 to 475 days old. The average latent period was 371.0 days.

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[8] Neufeld, A. H., and Collip, J. B., *Endocrinol.*, 1938, **23**, 735.

* This investigation was aided by a grant from the International Cancer Research Foundation.

† Research Fellow, the National Cancer Institute.

[1] Bonser, G. M., and Orr, J. W., *J. Path. and Bacteriol.*, 1939, **49**, 171.

[2] Strong, L. C., and Smith, G. M., *Yale J. Biol. and Med.*, 1939, **11**, 589.

[3] Murray, W. S., *Am. J. Cancer*, 1934, **20**, 573.

Most dilute brown mice died of leukosis if they were painted with methylcholanthrene solutions.[4] The carcinogen was applied to the unepilated skin twice weekly with a No. 6 camel's hair brush. The site was changed with each painting in the following order: head, left hind leg, right hind leg, left foreleg, right foreleg, sacral region, abdomen, interscapular region, anterior thorax. None of the male mice that were painted developed breast tumors. Thirteen of 65 breeding females of line 212 that were treated with methylcholanthrene had one or more mammary carcinomata. (Table I.) They appeared between the 106th and 268th day of life. The average latent period for these tumors was 181.3 days. The duration of painting varied. No breast cancer was found among 75 virgin females that were treated similarly.

The gross and microscopic appearance of the breast tumors was characteristic. The majority were typical adenocarcinomata which occasionally metastasized to the lungs. The adenoid structure was poorly retained in 2 tumors. Anaplastic cells grew in infiltrating masses and cords. Squamous metaplasia was found in some areas of one of the adenocarcinomata that followed painting with methylcholanthrene.

The number of observations does not permit any comparison of the breast tumor incidence in the 2 groups. The constant marked reduction in the latent period, however, is unquestionably significant. The reaction is analogous to that obtained with methylcholanthrene in mice subject to spontaneous lung tumors and in certain strains that develop spontaneous leukosis.

TABLE I.
Breast Tumors in 65 Breeding Female Line 212 Dilute Brown Mice Painted with Methylcholanthrene.

	Methylcholanthrene			
Mouse No.	Solvent	Concentration, %	Duration painting, days	Latent period breast tumor, days
1	Benzene	.5	65	106
2	Acetone	.25	90	145
3	Benzene	.5	91	157
4	Acetone	.25	90	164
5	Benzene	.5	57	166
6	Acetone	.25	109	187
7	Benzene	.25	144	190
8	Acetone	.25	106	191
9	Benzene	.5	84	198
10	,,	.5	97	208
11	,,	.5	97	208
12	Acetone	.25	154	229
13	Benzene	.5	90	268

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[4] Mider, G. B., and Morton, J. J., *Am. J. Cancer*, in press.

10984

Maintenance of Gestation in the Castrate Pregnant Rat with Androgens.*

R. R. GREENE AND M. W. BURRILL. (Introduced by A. C. Ivy.)

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

It is generally conceded that castration of the pregnant rat before the last few days of pregnancy results in termination of the pregnancy by resorption or abortion. Scipiades has demonstrated that pregnancy will continue after castration in this animal if testosterone or testosterone propionate are administered.[1] It is assumed that these androgens are able to maintain pregnancy because of their progesterone-like functions. Scipiades believes, however, that these androgens have a deleterious effect on the fetuses inasmuch as almost all of the offspring he obtained from treated mothers were born dead.

In the course of a study on the effects of androgens on embryonic sexual development, androgens have been administered to castrate pregnant rats in an attempt to eliminate the complicating factors of hormones produced by the maternal ovary. Some of these androgen-injected castrates have carried their pregnancies to term. Data concerning the effects of androgens on the course of pregnancy are presented here.

Thirty-one pregnant female rats were used in this study. The day of insemination was known for all animals. Castration was performed on the 8th to 14th day after insemination. The androgens were given daily in oil solution, starting on the day of castration, and continued to the 19th to 21st day of pregnancy. Parturition in the rats of our colony generally occurs on the 22nd day. Occasionally an animal delivers on the 21st day and rarely on the 23rd day. All animals were examined daily for vaginal bleeding and palpable progress of the pregnancy.

Twenty-five of these castrated pregnant rats were given testosterone propionate. In view of the fact that testosterone and androstenedione have been shown to have a weak progestational effect when tested in the rabbit,[2] these substances were used on the remaining 6 castrates. The number of animals used for this purpose was

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* Supported in part by a grant from the Josiah Macy, Jr., Foundation. The testosterone propionate (Perandren), testosterone, and androstenedione were furnished through the courtesy of Ciba Pharmaceutical Products.

[1] Scipiades, E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 242.

[2] Klein, M., and Parkes, A. S., *Proc. Roy. Soc. London*, 1937, **121**, 574.

TABLE I.

Substance	Day of castration	No. of animals	Daily dose, mg	No. resorbed	No. maintaining preg.	Avg No. of fetuses Living	Avg No. of fetuses Dead
Test. Prop.	8-10	10	5-10	10	0	—	—
	11	3	5	2	1	4	0
	11	3	10-15	1	2	3	2
	14	9*	5	0	6	5.7	1.5
Testosterone	12	2	20	0	2	7	0.5
Androstenedione	14	2	10	1	1	3	1
	14	2	15	0	2	1	2

* Includes 3 mothers who died on the 19th or 20th days. Their fetuses are not included in the table.

limited by the relatively tremendous amounts of these substances necessary (180.0 mg testosterone, or 80.0-120.0 mg androstenedione to each animal).

Most of the animals were killed on the 22nd day. In the group of 14 day castrates treated with testosterone propionate 3 animals bled vaginally late on the 21st day and accordingly were killed. Three more died of unknown causes on the 19th or 20th day. These animals had 11, 12, and 13 healthy appearing fetuses *in utero* which were not included in the table.

The dosages used and the results obtained are presented in tabular form (Table I).

It is evident that certain androgens (testosterone, testosterone propionate and androstenedione) can substitute for the ovaries in maintaining pregnancy in the rat. Their effectiveness in this regard, however, seems to be limited by a definite time factor. All 10 of the animals castrated before the 11th day resorbed; only half of the animals castrated on the 11th day carried to term, while most of the animals castrated after the 11th day carried to term. Whether this time factor is absolute or is due to insufficient or excessive dosage cannot be determined on the basis of the known data.

Scipiades' conclusions that androgens are injurious to the fetus are not substantiated by our results. The average number of living young in our testosterone and 14 day testosterone propionate groups compare quite favorably with the average number of living young from normal animals of our colony (7.6 for 35 consecutive deliveries with a range of 2 to 13). Scipiades also concluded that "the larger the dosage, the more deleterious is the effect on the fetus." Our own data does not support this conclusion. He gave dosages of 1.0, 2.0, and 5.0 mg daily to 11 castrated pregnant rats and obtained living young only from one rat which had been given the

5.0 mg dose. Consequently we do not believe that his data support such a conclusion. It is our impression that, in the animals castrated after the 14th day, at least, the opposite is probably true, *viz.*, the fetal deaths are due to insufficient dosage.

We are therefore able to confirm the findings of Scipiades that androgens maintain gestation in the castrate rat, but are unable to confirm his conclusion that these androgens are injurious to the fetus.

Summary and Conclusions. Testosterone, testosterone propionate and androstenedione maintained pregnancy with resulting living fetuses in rats castrated during the latter half of pregnancy.

10985 P

Effects of Selective Salivary Gland Extirpation upon Experimental Dental Caries in the Rat.*

Virgil D. Cheyne. (Introduced by G. H. Whipple.)

From the Division of Dental Research, Department of Pathology, School of Medicine and Dentistry, The University of Rochester, Rochester, N. Y.

The production of experimental tooth destruction seems to be one of the most valid methods by which oral pathology can be studied in the rat.[1] Although changes in caries-susceptibility have been obtained by the use of different diets it has not yet been adequately demonstrated whether the saliva plays a part in the observed effects. To test this relationship a procedure[2] for the extirpation of the salivary glands of rats has been reported which makes it possible to study the influence on the teeth of the removal of all or certain types of the salivary secretions. In this report various combinations of glands have been removed and the attending incidence of caries-susceptibility compared.

Experimental. A total of 88 rats were selected from the same Wistar breeding stock at 22 days of age and placed on two different diets. One of these (Hoppert, Webber, and Canniff[3]) was known to be productive of caries, and the other (regular stock[4]) has been found to produce little damage to the teeth.†

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* The investigation upon which this report is based was made possible by funds granted by the Carnegie Corporation of New York.

[1] Rosebury, T., *The Problem of Dental Caries, Dental Science and Dental Art* (S. Gordon, ed.), Philadelphia, Lea and Febiger, 1938.

[2] Cheyne, V. D., *J. D. Res.*, 1939, **18**, 457.

Groups of rats in each series were operated upon to remove the various salivary fractions. After 100 days the animals were chloroformed, the jaws separated, and all molar teeth examined by means of a fine-pointed explorer under a binocular microscope, magnification 30×. Each jaw was then split longitudinally, radiographed, and affected areas checked with the record. Several sagittal planes of jaw segments were examined histologically in ground and decalcified section to confirm the accuracy of the diagnosis.

Two methods of indexing were employed for differentiating the carious susceptibility between groups. The first made use of counting total carious teeth or cusps and indicating this factor as the "extent" difference. The second was based upon the postulate[5] that mechanical fracture of rat molars precedes most experimental carious destruction. It was assumed that a roughly uniform rate of fracture occurred in all groups on the same diet and the ratio of fractured teeth to carious teeth at the end of the experimental period represented differences in rate of carious involvement of these fractures. This ratio was designated as "activity", and for this reason the two types of lesions were recorded separately. The caries-index established in this manner denoted extent as well as activity of the carious process. Male and female differences were not sufficiently significant to warrant independent consideration in any group.

Results. Table I, series A and B, gives the essential details of the experimental procedures and results. The significant findings are as follows: (1) operated animals with totally removed serous saliva show a high incidence of caries-involvement, and in contrast to other groups, have a low incidence of fracture lesions (groups 2 and 6); (2) disturbance of glandular function by removal of other combinations of glands likewise results in an increased, although less marked, extent of caries; and in addition (although simple measurements of carious extent did not reveal this difference), (3) the animals show a greater caries-involvement of the fractures in direct proportion to the amount of serous saliva removed (groups 5, 3, 4, 6).

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[3] Hoppert, C. A., Webber, P. A., and Canniff, T. L., *J. D. Res.*, 1932, **12**, 161.

[4] Maynard, L. A., *Science*, 1930, **71**, 192.

† This stock diet has been used by us for 9 years and normal stock colonies bred and maintained on it have shown little or no caries.

[5] Bibby, B. G., and Sedwick, H. J., *J. D. Res.*, 1933, **13**, 429.

TABLE I.

Group	Exper. condition of animal	No. of animals	No. of molar teeth examined	No. of carious teeth involved		No. of carious cuspal involvements			No. of fractured teeth, per animal (x100)
				Total	per animal (x100)	Total	per animal (x100)	per tooth (x100)	
Series A: Tooth Involvements in Normal and Operated Animals on Stock Diet.									
I	Normal	39	468	6	15	7	18	1	26
II	Parotid and submaxillary glands removed	19	228	22	116	35	184	15	5
Series B: Tooth Involvements in Normal and Operated Animals on Hoppert, Webber and Canniff Diet.									
III	Normal	6	72	7	117	11	183	15	200
IV	Parotid glands removed	8	96	22	275	45	563	47	113
V	Submaxillary and major sublingual glands removed	8	96	18	225	47	588	49	263
VI	Parotid and submaxillary glands removed	8	96	81	1013	258	3225	269	38

Conclusions. Disturbance of normal salivary flow in the rat results in an increased incidence of experimental caries. The effect is most marked in rats with retained mucous saliva on a caries-producing diet and is in direct proportion to the amount of serous saliva removed. Caries production has even been observed in operated rats deprived of their serous saliva when fed a diet known to produce minimal dental destruction. The lower ratio of fracture to carious lesions in rats with such saliva indicates that the rate of development of carious lesions in these groups is very rapid.

10986

Responses of Hypophysectomized Immature Female Rats to Mare Serum Hormone.*

J. H. Leathem. (Introduced by P. E. Smith.)

From the Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York City.

Hypophysectomized mature and immature female rats have been shown to respond to mare serum hormone by a marked increase in ovarian weight.[1, 2] The ovaries of the mature rats contained stimulated follicles, corpora lutea and corpora lutea atretica.[3] However, in virtually every individual case the period of treatment, the dosage administered and the time elapsing between hypophysectomy and the start of treatment were varied.[1, 3] Due to the persistence of corpora lutea after hypophysectomy the mature rat does not afford a basis for accurate estimation of hormonal action. Recently Noble, *et al.*,[4] have shown that follicle stimulation, granulosa luteinization and interstitial cell development resulted from two different dosages of this material in hypophysectomized immature rats. The first dosage was started 24 hours after the operation and the second 10 days later.

The investigation here reported was undertaken to study the effects of varied doses of the mare serum hormone "Gonadin"† on

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* Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation, New York.

[1] Evans, H. M., Meyer, K., and Simpson, M. E., *Mem. Univ. Calif.*, 1933, **11**, 257.

[2] Smith, P. E., *J. Am. Med. Assn.*, 1935, **104**, 553.

[3] Hamburger, C., *Endokrinologie*, 1936, **17**, 8.

[4] Noble, R. L., Rowlands, I. W., Warwick, M. H., and Williams, P. C., *J. Endocrinology*, 1939, **1**, 22.

† Mare serum hormone, "Gonadin," was generously supplied by Doctor Donald Wonder, Cutter Laboratories.

hypophysectomized immature female rats that were all treated in the same manner. An interval of 5 days was allowed to elapse after hypophysectomy in all cases. The hormone was injected subcutaneously once daily for 5 days and the animals autopsied 24 hours after the last injection.

Sixty-three immature female rats ranging from 29 to 36 days of age were hypophysectomized, at which time the vaginal orifice was closed in all cases. Body weights at the time of operation ranged between 52 and 96 g and averaged 71 g, whereas the body weight at autopsy (10 days later) ranged between 53 and 92 g and averaged 67 g. All pituitary capsules were serially sectioned, stained with Masson trichrome and examined microscopically. Only data from completely hypophysectomized animals are presented. Weights of the adrenals, ovaries and uteri were taken at autopsy, and the ovaries were examined under the binocular microscope to ascertain grossly the response obtained. The ovaries of at least half of the animals on each dose level were sectioned serially.

Mare serum hormone was administered in 8 different total doses to groups of 5 to 10 animals each. The ovarian weight was found to increase with a corresponding increase in the amount of material injected. Ovaries of hypophysectomized controls after 10 days weighed 6 to 7 mg, whereas ovaries that weighed 250 mg were obtained with the largest dose of hormone employed. A greater effect would possibly have resulted had larger doses been used. Marked variation of ovarian weight was observed in most groups. Only 3 groups had less than 100% weight variation, 2 of which were the lowest doses tested (Table I).

Although ovarian weight was significantly increased with doses of 5, 10 and 12.5 r.u. the most pronounced effect observed with these doses was excessive thecal luteinization. A varied number of small follicles with or without antra were present, but in the ovaries of only

TABLE I.
Effects of Mare Serum Hormone "Gonadin" on Hypophysectomized Immature Female Rats.

Total dose, r.u.	No. of rats	Avg ovarian wt, mg	Range of ovarian wt, mg	Avg uterine wt, mg
5.0	5	12.1	9.5-15	27.6
10.0	6	21.5	18.5-27	50.8
12.5	9	28.4	17-45	118.0
20.0	6	48.3	38-63	128.0
25.0	9	84.3	50-137	119.4
30.0	9	104.4	74-190	143.0
40.0	10	145.4	92-241	120.7
50.0	9	184.1	91-250	148.3

3 of the 15 animals that received 10 and 12.5 r.u. were there follicles of medium size. A further increase of the dosage (20 r.u.) produced normal vesicular follicles of good size as well as luteinized thecal tissue. The heaviest ovaries in this group contained a large proportion of vesicular follicles. The closest approach to pure follicle stimulation was obtained with total doses of 25 and 30 r.u. The ovaries were primarily composed of large vesicular follicles which were fairly uniform in size and in which the granulosa and ova were normal. However, luteinized thecal tissue, although occurring in a very small amount, was nevertheless always present. The first evidence of granulosa luteinization was exhibited in the ovaries of rats that had received 40 r.u. and in a few cases corpora lutea had formed. Very large normal follicles formed a large part of most of these ovaries. The largest amount of hormone administered (50 r.u.) caused the formation of corpora lutea, many of which were corpora lutea atretica, follicles with thickened granulosa and some large normal vesicular follicles.

Summary and Conclusions. Cole[5] and Cartland and Nelson[6] reported their inability to secure evidence for the presence of more than a single principle in pregnant mare serum. However, Evans, *et al.*,[7] have indicated that this material can be fractionated with ammonium sulphate into FSH and ICSH as tested in hypophysectomized rats in a 72-hour period, but the details of the tests were not reported. It is well known that a varied ovarian response can be obtained with gonadotropins by altering the experimental conditions and the present experiments indicate the importance of dosage in the response elicited by mare serum hormone in hypophysectomized immature rats. In these experiments the predominant formation of thecal luteinization was observed with low doses of this material. Larger amounts of this hormone primarily caused the development of large vesicular follicles, whereas with a further increase of the dosage corpora lutea resulted. Large amounts of castrate urine extract also produced corpora lutea but this material produced only follicle stimulation in the absence of thecal luteinization in ovaries weighing as much as 80 mg.[8] Thus a definite difference in the gonadotropic activity of mare serum hormone and castrate urine extract can be cited as both materials were tested under identical experimental conditions.

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[5] Cole, H. H., *Am. J. Anat.*, 1936, **59**, 299.

[6] Cartland, G. F., and Nelson, J. W., *J. Biol. Chem.*, 1937, **119**, 59.

[7] Evans, H. M., Korpi, K., Simpson, M. E., and Pencharz, R. I., *Univ. Calif. Publ. Anat.*, 1936, **1**, 275.

[8] Tyndale, H. H., Levin, L., and Smith, P. E., *Am. J. Physiol.*, 1938, **124**, 174.

10987

Rate of Elimination of Nicotine by the Rabbit.

J. H. Weatherby. (Introduced by H. B. Haag.)
From the Department of Pharmacology, Medical College of Virginia, Richmond.

It is always advisable, and in some instances essential, for maximum therapeutic effectiveness and safety, to have fairly accurate information concerning the rate of elimination of drugs from the animal organism. For drugs which are excreted unchanged or in the form of some readily recognizable degradation product measurement of rate of elimination consists essentially of quantitative analyses of the excreta for the products sought. For other drugs whose fate in the body is less well understood the approach must be of a more indirect sort. Thus, with digitalis and the related glycosides, it is common practice to administer a fraction of the average lethal dose to suitable animals, then to ascertain after various time intervals how much additional drug must be administered to prove fatal. The difference between total amount of drug administered and average lethal dose represents the amount of drug eliminated from the organism in the period of time selected. For these drugs, which usually require from several days to a week or more for complete elimination, this method appears satisfactory. For certain others which seem to be practically completely eliminated within much shorter periods of time the method is not readily applicable. In his investigation of picrotoxin Dille[1] ascertained first the average convulsant dose of the drug for suitable test animals, then the amount of drug necessary after certain intervals of time to restore concentration within the organism to the convulsant level. Such an amount of picrotoxin Dille holds to represent the quantity eliminated during the interval since the initial convulsant dose was administered.

Kohn and Grimes[2] used a method somewhat similar to that of Dille in their studies on certain barbituric acid derivatives, and conclude that, at least for these substances, rate of elimination is proportional to the uneliminated residue. Mathematical formulae for calculating rate of elimination are presented by these authors. Others, among whom are Beck and Lendle,[3] Lendle,[4] Weese,[5] and

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[1] Dille, J. M., *J. Pharm. and Exp. Therap.*, 1938, **64**, 319.

[2] Kohn, R., and Grimes, C., *Anesthesia and Analgesia*, 1939, **18**, 139.

[3] Beck, A., and Lendle, L., *Arch. f. exp. Path. u. Pharmak.*, 1932, **164**, 188.

[4] Lendle, L., *Arch. f. exp. Path. u. Pharmak.*, 1933, **169**, 585.

[5] Weese, H., *Arch. f. exp. Path. u. Pharmak.*, 1936, **181**, 46.

Werner,[6] report studies in which essentially similar methods were used, varying in details according to individual needs.

The fact that relatively large portions of the populations of this as well as other countries frequently are exposed to nicotine through the use of tobacco, and the further fact that nicotine is a very interesting substance from the point of view of the pharmacologist, emphasize the necessity for additional information concerning this substance. Although several publications mention that relatively large quantities of nicotine are tolerated by the organism if administered sufficiently slowly—suggesting rapid elimination—no exact data on the subject have come to the attention of the author. The investigation of this subject was therefore undertaken. The term "elimination" is used here in its broadest sense; that is, to imply any process whereby the drug is rendered physiologically inactive, such as destruction, excretion, or conjugation. No attempt has been made in this investigation to ascertain the mechanism involved in elimination of nicotine.

Material and Methods. Male rabbits of mixed breeds weighing from 1200 to 1800 g were used in these experiments. Nicotine was administered as the acetate, although quantities are expressed in terms of the alkaloid. The solution injected contained 2 mg per cc, and all injections were made at the rate of 0.1 cc per second into marginal ear veins.

The various procedures described by the authors mentioned above presuppose a fairly accurate knowledge of the amount of drug required to produce a certain physiological reaction or end-point, whether this end-point be the onset of convulsions as with picrotoxin, paralysis of the respiratory center as with barbituric acid derivatives, or death of the animals as in the present investigation. Haag[7] found the average lethal dose (L.D. 50%) of nicotine for rabbits to be 6.2 mg per kg when administered intravenously. During the course of his investigation he observed that more consistent results were obtained when the rate of injection was made as nearly constant as possible. A rate of 0.1 cc per second proved to be satisfactory, so this precaution was observed throughout the present investigation. Sollmann and Hanzlik[8] report the M.L.D. as 10 mg per kg, but the percentage of animals succumbing to this dose was not mentioned.

Results. In the first series of experiments nicotine in doses of

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[6] Werner, H. W., *J. Pharm. and Exp. Therap.*, 1938, **63**, 39.

[7] Haag, H. B., *J. Lab. and Clin. Med.*, in press.

[8] Sollmann, T., and Hanzlik, P. J., *Fundamentals of Experimental Pharmacology*, Appendix G-I, p. 275, 1939.

1 mg per kg was administered at intervals of 1, 1½, 2, 3, 4, or 5 minutes until the animals died. The average amount of nicotine administered to the animals of a group less the average lethal dose (6.2 mg) indicates the average amount of nicotine eliminated by members of the group during the average period of time required for the animals to be killed. The amount eliminated divided by the number of minutes required gives the amount eliminated per minute. A second series of experiments was performed in which the size of the individual dose as well as the interval between doses was halved, although the amount administered per minute remained the same as in corresponding experiments of the first series. Thus, doses of 0.5 mg were administered at intervals of ½, 1, and 2 minutes. Also, 2 mg per kg were administered to another group at intervals of 2 minutes. Data obtained from these experiments are presented in Table I.

Fig. 1 shows the curve obtained by plotting rate of administration against rate of elimination. It will be noted that rate of elimination is lowest when rate of administration also is lowest. Under these conditions apparently the mechanism for eliminating nicotine is not functioning at its greatest capacity. As the rate of administration is increased there is a corresponding increase in rate of elimination until a maximum is reached. This maximum occurs when the rate of administration is approximately 0.5 mg per kg per minute, or 1 mg per 2 minutes. A further increase in rate of administration results in a decrease in rate of elimination. It will be seen also that results obtained from the second series of experiments agree satisfactorily with those from the first. An additional group of animals received 2 mg per 2 minutes, with the result that the apparent rate of elimination dropped considerably below that established by the first and

TABLE I.

Single dose, mg/kg	Dose interval, min	No. animals in group	Avg No. doses	Total adminis-tered, mg/kg	Total eliminated, mg/kg	Rate of elimination, mg/kg/min
1	1	20	8.3	8.3	2.1	.253
1	1½	20	12.0	12.0	5.8	.322
1	2	20	19.3	19.3	13.1	.339
1	3	10	23.7	23.7	17.5	.246
1	4	10	27.0	7.0	20.8	.193
1	5	10	31.6	31.6	25.4	.161
0.5	½	10	17.0	8.5	2.3	.271
0.5	1	10	33.3	16.67	10.47	.314
0.5	2	10	51.6	25.8	19.6	.190
2	2	10	3.6	7.2	1.0	.139

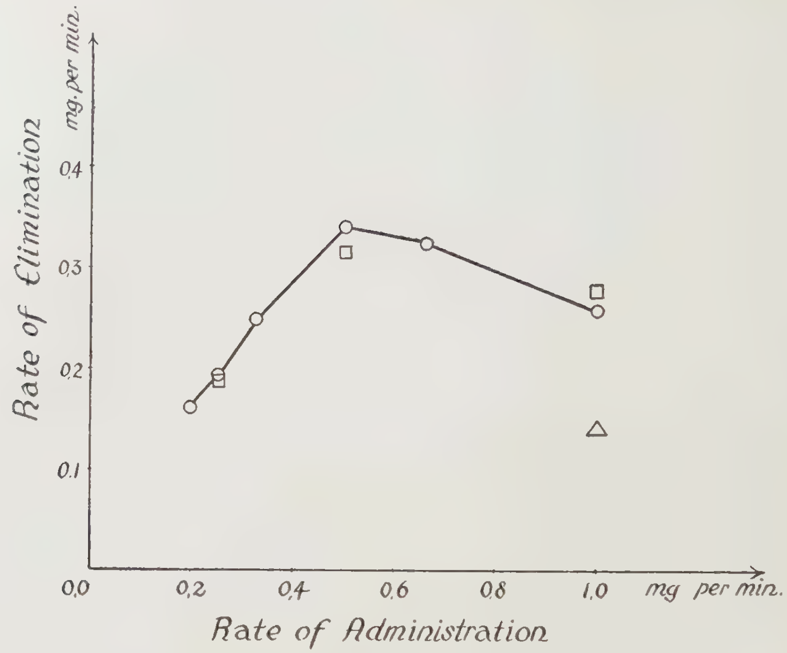

FIG. 1.

Relationship between Rate of Administration and Rate of Elimination.
○ = First Series. □ = Second Series. △ = 2 mg per 2 minutes.

second series of experiments. When one considers that such a dose represents nearly one-third the average lethal dose it is hardly surprising that such results were obtained. It is probable that these large doses so disturb physiological functions in the organism that elimination does not occur as rapidly as when doses of one-half the size or less are used.

Conclusions. When repeatedly administered in small doses the rate of elimination of nicotine seems to be proportional, within certain limits, to the rate of administration. When the optimum rate of administration is exceeded the rate of elimination is less than maximum, and ultimately reaches zero when the rate of administration is so rapid as to cause the end-point to be reached practically immediately.

10988 P

Effect of Thiamin Chloride on *Eimeria nieschulzi* Infection of the Rat.*

ELERY R. BECKER.

From Iowa State College, Ames, Iowa.

It was shown in previous experiments[1, 2] that wheat middlings and wheat bran supplements had the property of creating in the rat host a condition more favorable for the development of the coccidium *Eimeria nieschulzi* than the control ration, or even the control ration supplemented with white wheat flour. In a thorough recheck of the effects of wheat middlings (gray shorts), the following test ration (parts by weight) was used: beet sugar, 71; soy bean oil meal, expeller process, 10; casein, commercial, medium fineness, 10; normal salt mixture (Harris), 4; lard, 3; cod liver oil, 2. When 30 parts of wheat middlings were substituted for some of the sugar, casein, and soy bean oil meal, increased coccidium development, as indicated by counts of oöcysts passed by rats on the 2 rations, continued to be evident. Since, as was previously concluded, it seemed that amount of fiber could be ruled out as the causative factor, an investigation of the effects of extracts of wheat middlings was indicated.

Weighed amounts of wheat middlings were extracted by covering them with an excess of 95% ethyl alcohol and shaking several times daily for 3 days. The alcohol was recovered by filtration, and the process repeated twice. The test ration consisted of the control ration described above with the addition of alcoholic extract equivalent to 4 g of middlings dried onto each 10 g of the ration. This meant that each rat in the test series ate daily the material extracted by alcohol from about 4 g of wheat middlings. Animals were put on the rations when litters had attained a mean weight between 65 g and 85 g. After 10 days on the rations they were inoculated with 2000 sporulated oöcysts of *Eimeria nieschulzi,* and 3 days later with the same dosage. The weight gains recorded in Table I were those made during the first 17 days on the special rations, which included the first 7 days of the infection. The numbers of oöcysts recorded represent those passed during the ensuing period of oöcyst elimination, lasting about 10 days.

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* Aided by grants from the Industrial Science Research Institute, Iowa State College, and the American Academy of Arts and Sciences.

[1] Becker, E. R., and Derbyshire, R. C., *Ia. St. Col. J. Sci.*, 1938, **12**, 211.

[2] Becker, E. R., and Waters, P. C., *Ibid.*, 1939, **13**, 243.

TABLE I.
Oöcyst Counts and Weight Gains for Rats Receiving Alcoholic Extract of Wheat Middlings and Thiamin Chloride Compared with Controls.

		Test Series			Reference Series		
Group	Supplement	No. rats	Mean wt, gain	Mean millions of oöcysts	No. rats	Mean wt, gain	Mean millions of oöcysts
1	Extract mids	3	55	164	3	37	201
2	,, ,,	3	46	55	3	24	183
3	,, ,,	3	39	78	3	20	156
4	Thiamin chloride	8	21	35	8	16	67
5	,, ,,	3	31	44	3	21	51
6	,, ,,	4	28	61	4	20	120

Table I shows that there was a striking difference in the quantitative character of the infections in the groups of rats on the two rations. Since alcoholic extracts of cereal products usually contain considerable amounts of vitamin B, there was a possibility of it being the factor concerned in limiting the infection. Accordingly, the experiments were repeated with the exception that the test series received 10 micrograms of thiamin chloride per rat as a daily supplement in place of the alcoholic extract of middlings. The table shows that in this case also there was a decided difference in the counts for the two lots. Conversely, the test series made greater weight gains when either alcoholic extract or pure crystals were used as the source of vitamin B.

The experiments indicate that vitamin B_1 (thiamin chloride) exerts a restraining influence on the development of the coccidium *Eimeria nieschulzi* in its rat host.

10989

Relation Between Prothrombin Concentration and Retraction of the Blood Clot.*

P. M. AGGELER AND S. P. LUCIA.

From the Division of Medicine, University of California Medical School, San Francisco, California.

It has long been known that one of the salient features of the bleeding tendency in obstructive jaundice is a fragile, non-retractile blood clot. Quick[1, 2] showed that the essential defect in the plasma of patients with the bleeding tendency of obstructive jaundice was a deficiency of prothrombin. Boyce and McFetridge[3, 4] found that in normal subjects the volume of serum expressed from the blood clot in 4 hours at room temperature was equal to half the total volume of the specimen. In patients with obstructive jaundice the volume was significantly less. They expressed this relationship as the serum volume index, in which the volume of serum is divided by half the total volume of the specimen. The index is corrected for deviations from the normal erythrocyte count. The serum volume index for normal subjects was 1.00; the highest index at which a patient with obstructive jaundice bled was 0.71; the lowest index at which hemorrhage did not occur was 0.78. Mettier and Witts express the measurement of clot retraction as the clot retraction index. In their test[5] the volume of serum expressed from the clot in one hour at 37°C is divided by the total volume of the specimen; to this is added the packed cell volume and the final result is divided by two. In normal subjects the clot retraction index ranged from 38 to 50. We have used both the clot retraction index and serum volume index. Although the trend of the results with either test is the same, the clot retraction index is perhaps a better measure of this phenomenon, because it eliminates variables due to fluctuation in temperature and mean corpuscular volume.

It is obvious that the degree of retraction of the blood clot depends upon at least 2 factors: the amount of prothrombin and the amount

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* Assisted by a grant from the Christine Breon Fund.

[1] Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. M. Sc.*, 1935, **190**, 501.

[2] Quick, A. J., *J. A. M. A.*, 1938, **110**, 1658.

[3] Boyce, F. F., and McFetridge, E. M., *J. Lab. and Clin. Med.*, 1937, **23**, 202.

[4] Boyce, F. F., and McFetridge, E. M., *New Orleans Med. and Surg. J.*, 1939, **91**, 357.

[5] Mettier, S. R., and Witts, L. J., in press, personal communication.

TABLE I.

Samples	No. of tests	Prothrombin Concentration Mean	Prothrombin Concentration Sigma	Clot Retraction Index Mean	Clot Retraction Index Sigma	Correlation Coefficient
A	85	72	37	38	5	.450 ± .09
B	44	80	25	39	6	.634 ± .09
C	41	78	25	39	6	.641 ± .09

of thromboplastin, the volume of the corpuscles under ordinary circumstances playing a minor rôle. The amount of prothrombin can be estimated by the method of Quick.[2] The amount of thromboplastin in a sample of blood is conditioned by the number and quality of the platelets, and the amount of tissue juice expressed from traumatized tissues.

We have recorded the clot retraction index and prothrombin concentration of 85 samples of blood. The blood was obtained from normal subjects and from patients suffering from obstructive jaundice and from a variety of diseases affecting the liver. Patients with hemophilia and thrombocytopenic purpura were excluded. Calculated statistical values are shown in Table I.

The Prothrombin Concentration. By the terms of this test the variable factor apparently is the quantity or quality of the prothrombin contained in the test sample. The test can be accurately performed; our experimental error in repeated estimations of the prothrombin concentration in the same subject is not greater than 5%. It is a delicate test, as is apparent from the value of the spread around the mean.

The Clot Retraction Index. In this test there are at least 2 variable factors, one the prothrombin content, and the other the quantity of thromboplastin in the blood sample. This test is less delicate as can be seen from the narrowness of the spread of the values around the mean. When done at the same time and on the same sample of blood which is used for the prothrombin time, the fluctuation in the clot retraction index for a given prothrombin concentration will apparently be due to fluctuations in the quantity of thromboplastin.

Correlation Coefficients. Sample A—85 tests were performed on samples of blood obtained from 44 persons including both normal and pathologic individuals. Of these, 50 samples were obtained from 9 individuals before and after recovery from prothrombinemia. The correlation coefficient of 0.450 ± 0.09 is positive and significant, and indicates that on the average as the prothrombin concentration is lowered, the clot retraction index is also lowered, that is, the 2 factors fluctuate in the same direction simultaneously.

Sample B—Because sample A was weighted by 50 tests from 9 individuals, it was decided to select only one reading (the lowest prothrombin concentration) from each individual, normal or pathologic. The final sample included 44 tests. The correlation coefficient of 0.634 ± 0.09 indicates to a greater degree the same relationship found in sample A.

Sample C—A further reduction of sample B made by eliminating the tests on the three normal individuals ($N = 41$) slightly raised the correlation coefficient ($r = 0.641 \pm 0.09$).

An example of the close correlation, which may be observed in some patients, between the prothrombin concentration and the clot retraction index is given in Fig. 1.

Clinical bleeding from mucous membranes occurred when the prothrombin concentration dropped to 10% and the clot retraction index to 30. When the prothrombinemia was corrected by the parenteral administration of Vitamin K, both the prothrombin concentration and clot retraction index returned to normal.

The following example, however, illustrates the error which may be

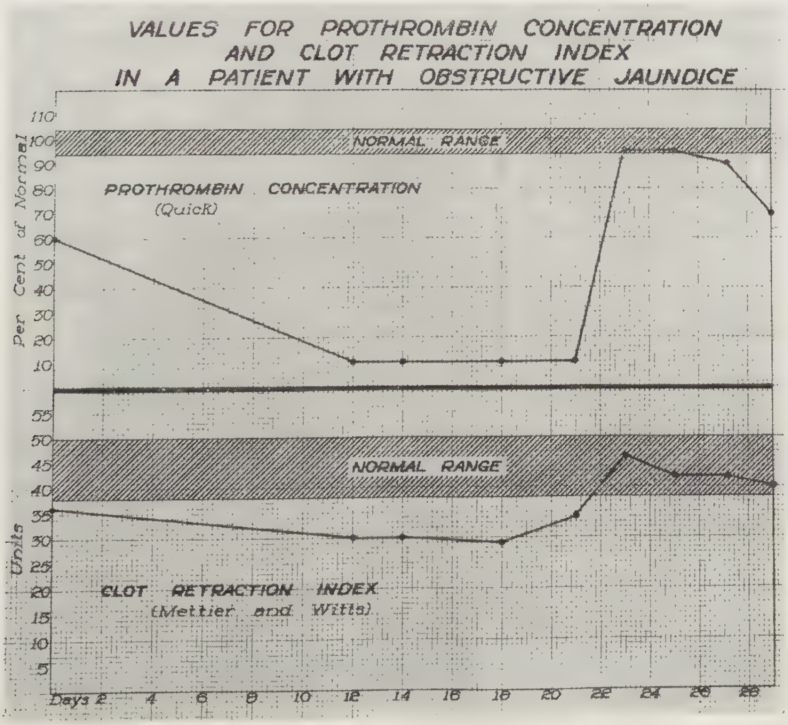

FIG. 1.

made in ascribing to prothrombinemia alone the deficient clot retraction observed in some cases of liver damage. A patient entered the hospital because of recurrent icterus. She was found to have marked hepato-splenomegaly. Icteric index 25. Whole blood coagulation time 7½ minutes. Prothrombin concentration 100%. Clot retraction index 31. The capillary fragility was markedly increased, and the platelet count was 65,000 mm^3.

It is obvious that the deficiency in clot retraction was probably due to decreased thromboplastin and not to any alteration in prothrombin concentration.

Summary. A relationship has been established between the prothrombin concentration and the clot retraction index. This should not be interpreted as a direct cause and effect relationship, inasmuch as other variables may influence both tests in common. In the case of the clot retraction index, thromboplastin, as well as prothrombin plays an important rôle in the reaction. The variation of the prothrombin concentration, when evaluated in terms of diagnosis, appears to be a more sensitive index than the clot retraction index.

10990

Age and the Rate of Venous Enlargement under Increased Venous Pressure.*

T. ADDIS AND W. LEW.

From the Department of Medicine, Stanford University Medical School, San Francisco.

By tying the vena cava of rats above the entrance of the renal veins an intense venous congestion is produced in the kidneys. The formation of urine stops and the blood urea concentration rapidly rises. In surviving animals a small amount of dilute urine containing a high concentration of protein and many renal failure casts is excreted after 24 hours, but the blood urea concentration still rises until 48 hours after operation it is more than 300 mg per 100 cc. Thereafter the volume of urine increases, the concentration of urea rises in the urine and falls in the blood, the proteinuria diminishes, the casts disappear and in a week after operation there is no evidence of any renal lesion.

The mechanism of the process of restoration is dependent on the

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* This work was aided by a grant from the Rockefeller Foundation.

rate of enlargement of venous connections between veins draining into the vena cava and veins in the pelvis that connect with veins in the abdominal wall returning blood to the heart above the ligature on the vena cava. The progressive enlargement of the veins in the abdominal wall can be seen through the peritoneum when the abdominal cavity of rats is opened at successive intervals of time after ligation of the vena cava. After the operation the blood from the renal veins has to run down instead of up the vena cava, and the venous pressure in the kidney falls as the connecting veins enlarge and allow the blood to return to the heart. When the rate of venous enlargement is sufficiently rapid the congestion in the kidney diminishes and urine formation is reëstablished. When the rate is slower the animal dies of uremia before the venous pressure has fallen sufficiently to make adequate excretion possible.

In the course of experimental studies on this acute reversible form of uremia[1, 2] we have had occasion to do many control operations on ♀ albino rats, all reared on a stock diet that contains 18% protein, and all maintained under the same conditions. The only variable (aside from the fact that in the groups of 6 months or more some had borne litters) was age. Table I shows the effect of age at the time of operation on the percentage mortality rate within 5 days after the ligation of the vena cava.

TABLE I.

Age in months	No. of rats	% mortality
1	52	0
3	55	3.6
6	211	5.2
12	169	17.8
18	36	30.6
23 (avg)	58	90.0

Conclusions. 1. After ligation of the vena cava above the entrance of the renal veins a plot of the logarithms of the percentage mortality on age seems to indicate that they are a linear function of the age at the time of operation. 2. It is suggested that the above relation may be the result of a decrease in the rate of venous enlargement under increased pressure as age advances.

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[1] Addis, T., and Raulston, B. O., *Trans. Assn. Am. Physic.*, 1930, **45**, 318.

[2] Addis, T., and Lew, W., *J. Clin. Invest.*, 1939, **18**, 773.

10991

Prothrombin, Thromboplastin, and Thrombin: Quantitative Interrelationships.*

Edwin T. Mertz, Walter H. Seegers and H. P. Smith.
From the Department of Pathology, State University of Iowa, Iowa City.

We wish to present data concerning the relative amounts of prothrombin, thromboplastin, and calcium which are needed to form a given amount of thrombin. One obstacle to such a study has always been that prothrombin and thromboplastin are accompanied in nature by antithrombin. Unless this antithrombin is carefully removed, the thrombin is inactivated almost as rapidly as it is formed, and it is impossible to obtain satisfactory data concerning the amount of thrombin produced.

With technics recently developed in this laboratory we can prepare prothrombin and thromboplastin which are highly potent and are free of antithrombin; but while using these preparations we encountered a second obstacle which had not been anticipated. It was found that on incubating prothrombin, thromboplastin and calcium, there is promptly formed a substance which inactivates prothrombin, and thus decreases the eventual yield of thrombin.[1] This destructive agent was not present in the prothrombin, thromboplastin, nor in the calcium. It is evident that either the destructive agent is thrombin itself, or that it is some other substance which is formed under similar conditions.

In the present study the inactivation of prothrombin is still evident, but by making due corrections, it appears that the formation of thrombin from prothrombin and thromboplastin follows simple laws of proportion. This finding eliminates support for the old concept that thromboplastin is a traditional enzyme, capable of converting unlimited quantities of prothrombin into thrombin. This work also supplements older work on prothrombin and thromboplastin which until now has been on a qualitative basis.[2, 3]

Experimental Procedure. The purified thromboplastin solution was prepared from beef lung as previously described.[1] The prepa-

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* Aided by a grant from the John and Mary R. Markle Foundation. Funds for two research assistants were supplied by the Graduate College, State University of Iowa.

[1] Mertz, E. T., Seegers, W. H., and Smith, H. P., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 657.

[2] Eagle, H., *J. Gen. Physiol.*, 1935, **18**, 531.

[3] Ferguson, J. H., *Am. J. Physiol.*, 1938, **123**, 341.

ration of thromboplastin thus obtained contains approximately 2.5% organic solids. In the present experiments it was diluted 11-fold with calcium-saline (0.9% NaCl + 0.15% $Ca(NO_3)_2$) prior to use.

In order to determine quantitatively the effect of thromboplastin, it is essential that the prothrombin used be entirely free of thromboplastin. Technically this result is not readily achieved, and most of our prothrombin preparations[4] form a limited amount of thrombin when calcium is added. In the present experiments, however, we used only those samples of prothrombin which did not react with calcium unless thromboplastin was added also.

It was also important for these studies that antithrombin be eliminated as completely as possible from the reacting mixtures. All solutions of prothrombin and of thromboplastin were tested in advance by being mixed with measured amounts of thrombin. These preparations were then accepted for use provided they produced no detectable destruction of thrombin in 10 hours.

The prothrombin was made up in a solution with NaCl (0.9%) and $Ca(NO_3)_2$ (0.15%) which was diluted with 5% of its volume of imidazole buffer[1] before the prothrombin was added. The amount of calcium present was found to be physiologically optimal. An increase

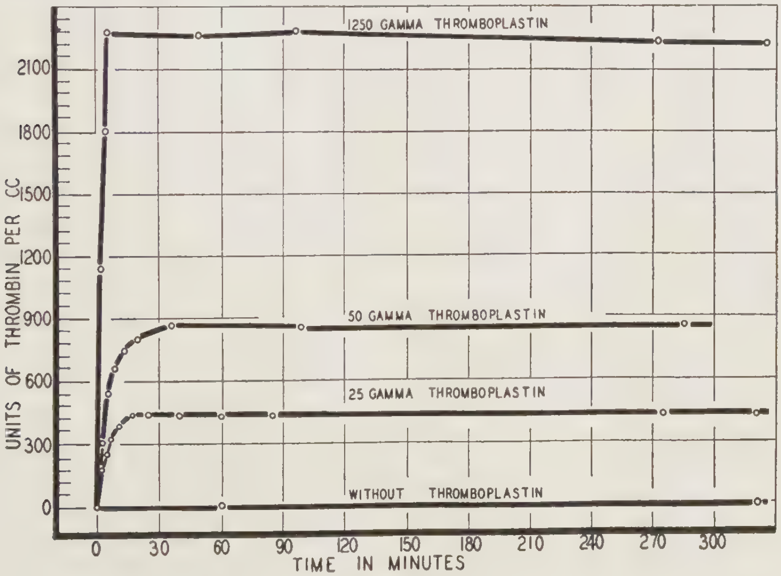

FIG. 1.

Rate of thrombin formation with small and with large quantities of thromboplastin. Initial prothrombin concentration was 2300 units per cc.

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[4] Seegers, W. H., Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Biol. Chem.*, 1938, **123**, 751.

or decrease of 20% had no effect on the results. The prothrombin solution used contained 4% prothrombin, and had a thrombin titer of approximately 2200 units of prothrombin per cc (*i. e.,* on being diluted 2200-fold, the thrombin formed from it clots fibrinogen in a standard interval of 15 seconds).

Results. Figure 1 shows the result of treating a large amount of prothrombin with calcium and variable quantities of thromboplastin. With 25 gamma of thromboplastin, the thrombin titer rose within 25 minutes to the 400 unit level. It then remained at this low level for many hours. This experiment shows that thromboplastin does not convert unlimited quantities of prothrombin into thrombin.

By increasing the amount of thromboplastin enormously, the relationships are reversed. Under these circumstances, we observed that prothrombin also has a sharply limited capacity to produce thrombin. Thus, on adding 1250 gamma of thromboplastin, there occurred a rapid production of 2300 units of thrombin. Other experiments, not shown on the chart, showed that further increase in thromboplastin did not increase the yield of thrombin beyond this point.

Figure 1 also shows the results obtained with 50 gamma of thromboplastin. The yield of thrombin in this case was almost exactly twice as great as when 25 gamma were used. This suggests a rela-

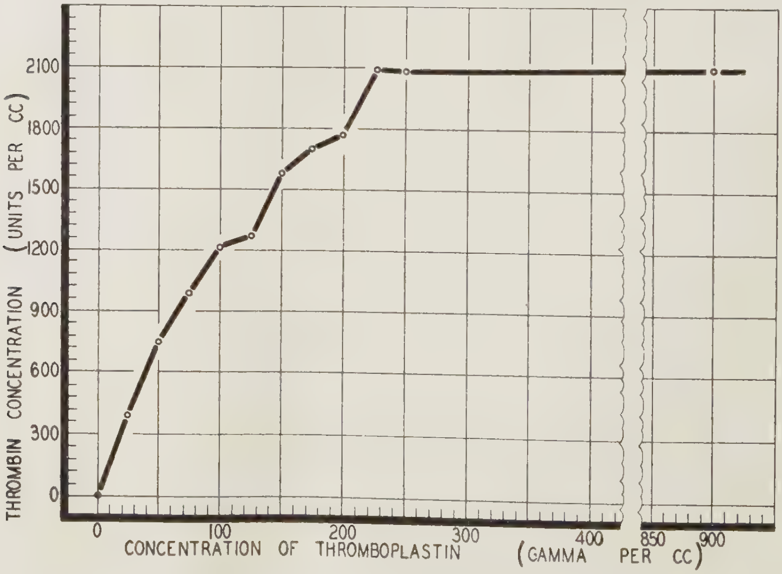

FIG. 2.

Total thrombin production with varying quantities of thromboplastin. In all cases the initial prothrombin concentration was 2100 units per cc. The curve gives the thrombin concentration at the end of 10 hours.

tionship of direct proportionality, and for the purpose of studying this further, a larger series of tests was made, the results of which are given in Fig. 2. Varying amounts of thromboplastin were employed, and in each case, sufficient time was allowed for the thrombin titer to reach its maximum. These maxima are presented in the chart as ordinates; the abscissae correspond in each case to the amount of thromboplastin used. The maximum yield of thrombin, 2100 units per cubic centimeter, was obtained by adding 225 gamma of thromboplastin. The individual protocols of these experiments showed that larger amounts of thromboplastin increased somewhat the reaction rate, but not the amount produced.

The curve leading up to the 2100 unit level followed quite closely the path of a straight line throughout the first half of its course. The second half deviates definitely to the right, giving the inflection point at the top a definitely rounded appearance. Suspecting that this deviation from the course of a straight line might be due to complicating factors, we conducted studies which led to the discovery mentioned above that thrombin itself appears to destroy a part of the prothrombin, and thus prevents the thrombin titer from being as great as might otherwise be the case.

Figure 3 illustrates the degree to which prothrombin inactivation occurs. To a solution containing 2300 units of prothrombin were added 16 gamma of thromboplastin. The thrombin titer rose within

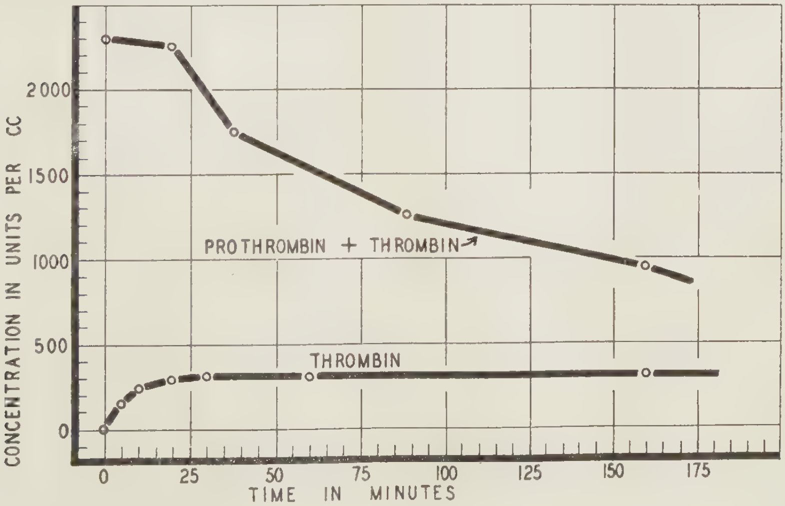

FIG. 3.

Inactivation of unconverted prothrombin. The prothrombin activity was measured as the increment of thrombin produced in 6 minutes by the addition of a large excess (1250 gamma) of thromboplastin.

25 minutes to the 300 unit level, after which the titer remained unchanged. This formation of thrombin involved, theoretically, a corresponding fall in the prothrombin titer but no fall in the combined titer of the two. That the combined titer did fall is shown in the figure and is due to inactivation of prothrombin.[1] This inactivation did not eliminate the supply of prothrombin for many hours, but did reduce the supply markedly during the period illustrated in the figure. In this particular experiment, it is evident that the supply of prothrombin was adequate to exhaust completely the thrombin-forming power of the thromboplastin. In the lower portion of the curve of Fig. 2, there is also an excess of active prothrombin, and the thrombin values approached very closely the maximum theoretical converting power of the thromboplastin used. The same can be said of the horizontal portion of the curve at the top of Fig. 2. In this portion of the curve, thromboplastin was present in such great excess that the reaction was driven rapidly to completion and the time which elapsed was too brief to permit any consequential changes in the reactivity of the prothrombin. However, in the upper half of the ascending curve (1200-2100 units thrombin), the reaction proceeded more slowly and the inactivation of prothrombin was such that the full titer of thrombin could not be obtained. In experiments not recorded here, we have shown that under these conditions, reactive thromboplastin still exists in the mixture. On adding additional quantities of prothrombin, further amounts of thrombin are formed and the entire ascending portion of the curve approximates that of a straight line.

We have conducted many other experiments of this general type, but differing in having a constant quantity of thromboplastin and variable quantities of prothrombin. The curves have all been similar in form to those shown in Fig. 2. There is, in each case, an intermediate zone in which a measurable quantity of prothrombin disintegrates and must be replaced, if one is to obtain the theoretical yield of thrombin. With this correction, the experiments confirm the law of proportionality developed in connection with Fig. 2.

The prothrombin solution used in the above experiments contained 40 mg prothrombin per cc. By correcting Fig. 2 for prothrombin inactivation, it is evident that approximately 0.15 mg of thromboplastin were equivalent to this amount of prothrombin. This implies a utilization ratio of approximately 265 to 1. With further purification of prothrombin and thromboplastin, these data are subject to revision. The figure is one which must be given consideration in conducting further work on the mechanism of thrombin formation.

Finally, we wish to point out that the rule of proportionality permits us, for the first time, to lay the groundwork for a quantitative method for the titration of thromboplastin. By mixing the unknown material with an excess of purified prothrombin, one can bring about the rapid development of thrombin in the solution. The titer of this thrombin solution is directly proportional to the amount of thromboplastin present. This method is analogous to the one developed and used in this laboratory for the titration of prothrombin.

Summary. Thromboplastin is consumed when it reacts with prothrombin in the presence of calcium ion. This finding eliminates support for the old concept that thromboplastin is a traditional enzyme, capable of converting unlimited quantities of prothrombin into thrombin. Evidence is presented which shows that the quantity of thrombin produced from an excess of prothrombin is directly proportional to the quantity of thromboplastin added to the reaction mixture. When, on the other hand, thromboplastin is present in excess, the amount of thrombin formed is proportional to the amount of prothrombin added.

10992

Occurrence of the Pellagra-Like Syndrome in Range Chicks.

HERMAN C. MASON. (Introduced by Lloyd Arnold.)

From the University of Illinois College of Medicine, Department of Bacteriology and Public Health, Chicago.

In chick virus transmission experiments it is sometimes necessary to use range chicks hatched away from the laboratory. The presence or absence of borderline nervous symptoms are often neither observed nor detected as part of the experimental procedure. The use of commercial rations is usually unsatisfactory in relieving cannibalism,[1] and other findings such as delayed prothrombin clotting values may occur in supposedly normal chicks.[2, 3]

This note summarizes the results of an investigation of the occurrence of the pellagra-like syndrome in range chicks maintained on an adequate ration.

In over a thousand pullorum-free White Leghorn chicks maintained since hatching (April 12) at a farm range, over 200 presented symptoms of which a laboratory report diagnosed as "*B. coli* bacillosis." Visiting the farm and recognizing pellagra-like symptoms of Ringrose, Norris and Heuser[4] in varying degrees of severity on eyes, beaks, toes and feathering, present in the 85 survivors at the time (May 20), they were then removed to the laboratory. The chicks had been maintained on the following hatching ration prepared carefully on a commercial scale per 2600 lbs:

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[1] Bass, C. C., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 488.

[2] Schønheyder, F., *Am. J. Physiol.*, 1938, **123**, 348.

[3] Mason, H. C., and Smith, M. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 583.

Fish meal	130
Milk	200
Meat scraps	100
Soy bean meal	100
Whey	50
Barley	120
Ground oats	400
Bran	400
Corn meal	400
Middlings	400
Dehyd. alf. meal	200
Bone meal	25
CaCO, MnSO	35
Salt-iodine	25
Cod liver oil XX	15

The diet and water were supplied *ad libitum*. The chicks were permitted to range freely from the shelter.

In the laboratory the chicks were placed on an approved commercial ration fairly satisfactory for chicks but not for adults. Neither cannibalism nor paralysis was observed on crowding. Postmortem examination of the chicks that died during the first few days in the laboratory revealed gizzard erosions and duodenal inflammation. Food residues were almost absent. The liver showed a deep dirty-yellowish color, while the spleen and kidneys showed small amounts of inflammation. Gas was present in the entire intestinal tract. These findings probably account for the diagnosis of "*B. coli* bacillosis", which was confirmed in the severely affected chicks, and is a common postmortem finding. The sacrifice of several mildly affected chicks did not reveal the bacillosis. The majority of the chicks recovered uneventfully in from 2 to 4 weeks, although the rate of recovery was not uniform, but progressed rapidly for those mildly affected.

The weights and sizes of the chicks were found to be extremely variable and gave no clue as to the defect of metabolism since the majority of the chicks on the ration were observed to be free of the symptoms. However, the chicks that were retarded in growth presented the most severe external lesions at the eyes, mouth, feet, and feathers identical to chicks maintained on the "F" ration of Ringrose, *et. al.* The margins of the eyelids were granular, and firmly stuck together by exudate. No xerophthalmia or cataracts were found. Crusty scales were present at the corners of the mouth. The outer layers of skin on the bottoms of the feet and between the toes were discolored, fissured and pealed wherever cracked. Feathering was markedly retarded and the feathers were dirty, rough and staring with spotty loss.

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[4] Ringrose, A., Norris, L., and Heuser, G., *Poultry Sci.*, 1930-31, **10**, 166.

Substances contained in the vitamin B complex have been shown to be of essential importance for nervous system protection. Shattuck[5] has pointed out that failure to assimilate and utilize these substances may be as potent a cause of neurologic pathologic changes as is the failure to ingest adequate amounts of vitamin.

Since results with nicotinic acid in chicks and rats have been negative,[6, 7, 8] it is probable and not at all unlikely that the chick's resistance to pellagra may depend on more than one factor.[9] The fact that the chicks recovered on the laboratory ration may not mean that the filtrate factor[10] was not lacking, but that the need for it was diminished, although the range ration meat scrap content should have contained sufficient quantity of the factor.

It may be concluded from these observations that the occurrence of the pellagra-like syndrome in these range chicks maintained on a supposedly adequate ration is not significantly different from that observed experimentally in the laboratory and is of great interest bacteriologically, since the *B. coli* bacillosis was not a consistent finding. The significance of these results is to indicate further the lack of relationship between the paralytic factor of Norris[11] and Bethke[12] and the growth-promoting factors in the chick. It also points to the existence of borderline deficiency syndromes in range chicks maintained on supposedly adequate rations, and indicates that the factors of nutrition and metabolic disturbance, when the preventive reserve is low, play an important etiologic rôle in similar conditions in other range flocks.*

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[5] Shattuck, G. C., *J. A. M. A.*, 1938, **111**, 1729.

[6] Cook, C. A., Clarke, M. F., and Light, A. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 514.

[7] Macrae, R. F., and Edgar, C. E., *Biochem. J.*, 1937, **31**, 2225.

[8] Emerson, G. A., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 195.

[9] Mitchell, H. H., *J. Biol. Chem.*, 1919, **40**, 399.

[10] Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, 1936, **115**, 557.

[11] Norris, L. C., Heuser, G. F., and Wilgus, H. S., *Poultry Sci.*, 1930, **9**, 133.

[12] Bethke, R. M., Record, P. R., and Kennard, D. C., *Poultry Sci.*, 1931, **10**, 355.

* After this manuscript was ready for publication an article by Mohammad, Emerson, Emerson and Evans on "Multiple Nature of the Rat 'Filtrate Factor'—a Component of Vitamin B_2" appeared in *Science*, October 20, 1939, issue.

10993

Response of the Human Lens to a Sudden, Startling Stimulus.

M. W. Morgan, Jr., and J. M. D. Olmsted

From the Division of Physiology, University of California Medical School, Berkeley, California.

It has been shown, by means of skiascopy, that the rabbit's eye becomes more hypermetropic when the animal is startled or excited.[1] This reaction has now been found to take place in man under suitable conditions.

Observations have been made on 54 different human subjects. The subject's eyes were first "neutralized" by skiascopy, then while the "against motion" of the shadow was being observed, a slight electric shock from an induction coil was administered through electrodes held between the subject's thumb and forefinger. The shock was of sufficient strength to be distinctly felt but not strong enough to cause distress. The sending of the shock was controlled by the foot of the observer, the subject being unaware when the contact switch was being thrown in.

In 37 of the 54 subjects the eye under observation became more hypermetropic in response to the electric shock as shown by the sudden change of the skiascopic shadow from "against motion" to "with motion". In all except 2 the change was only a momentary one and quantitative measurement of it was impossible. In 2 subjects, however, the reaction was more sluggish and therefore measurable. In one the change was 0.50 D, and in the other 0.75 D. Judging from the speed of the shadow motion, the observer (M.W.M.) inferred that in all the subjects who showed the reaction, except the 2 just mentioned, the change was approximately 0.25 D.

One-third of the subjects did not show a reaction of the lens to the electric shock to the fingers, although in each case the pupil dilated. It was thought that this type of stimulus might be inadequate for some persons but a different stimulus might cause the reaction to appear. With the assistance of Dr. N. W. Shock and Mr. J. Pack, one subject of each group was subjected to a long series of auditory stimuli, such as a musical note from an oscillator, sounds from a shrill siren, a small bell, an automobile horn, a shot from a cap pistol, and a loud snap of a flat spring against a wooden panel. At the time that the shadow in the eye was being observed, change in size of the pupil was also noted and records were taken of skin resistance (psychogalvanic reflex), pulse rate, and foot volume, *i. e.*, reflexes known to involve the sympathetic nervous system.

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[1] Olmsted, J. M. D., and Morgan, M. W., Jr., *Am. J. Physiol.*, 1939, **127**, 602.

In the subject who had shown no lens response to the electric shock on the finger (a laboratory technician who is accustomed to receiving such shocks in repairing electrical apparatus) the pupil dilated to each change of auditory stimulus but not to a moderate one after being several times repeated; the skin resistance changed to 60% of the stimuli, the heart rate to 26%, the foot volume to 20%, and the lens to 33%. The siren and snap of the spring were the most effective stimuli, the automobile horn and cap pistol next. This shows that the lens response will occur with one type of stimulus although it may not with another.

In the subject (J.M.D.O.) whose lens responded to finger shock, the pupil dilated and the skin resistance gave large variations upon each auditory stimulus, no matter how many times repeated; the pulse responded to 30% of the stimuli, the foot volume to 26%, and the lens to 37%.

In both subjects changes in pulse rate, foot volume and lens appeared independently of one another but in about the same proportion of trials. The more sudden and startling the stimulus, the greater the likelihood of the appearance of more than one of these responses. The fact that all these reactions, except the lens response, are known to be effects of sympathetic stimulation strengthens the view previously stated that increase in hypermetropia attendant upon startling the subject must also be due to sympathetic stimulation.

Summary. Appropriate sudden stimuli, such as an electric shock on the hand or a loud noise, causes the human lens to become momentarily more hypermetropic. Since this effect is always accompanied by dilation of the pupil and may be accompanied by change in skin resistance, increase in heart rate, and decrease in foot volume, it, too, is thought to be a result of sympathetic stimulation.

10994

Influence of Light Rays on Coagulation of Plasma and Fibrinogen.

LUDWIG PINCUSSEN. (Introduced by William H. Welker.)

From the Department of Physiological Chemistry, University of Illinois, College of Medicine.

It has been shown by many experiments that proteins are highly susceptible to the action of ultraviolet rays.[1, 2] Mond[3] claims an increase in stability of globulin and fibrinogen solutions when irradiated with U. V., which may correspond to the behavior of a well defined colloid, congorubin.[1] Howell[4] was able to demonstrate delayed coagulation of fibrinogen solutions and when hematoporphyrin was added to such solution before irradiation no coagulation occurred.

The experiments recorded in this paper are concerned with: (1) comparison of various kinds of light on the coagulation-time; (2) comparison of the coagulation time of plasma on one hand and solutions of fibrinogen on the other.

Fresh beef blood from full grown animals was oxalated and the plasma separated by centrifugation. A portion of the plasma was used to prepare fibrinogen according to the method described by Hektoen and Welker.[5]

The light sources used for the experiments were: (1) The high pressure mercury-quartz lamp, characterized by its very effective U. V. spectrum with only a few visible lines. (2) The carbon-arc lamp, used with carbons containing various metals to produce different kinds of continuous spectra.

(a) The C-carbon, producing besides visible rays a very large quantity of ultraviolet.

(b) The SS-carbon, giving much energy in the infra-red, visible and near ultraviolet regions but a very low output in short wave U.V.

(c) The E-carbon, emitting large quantities of visible and infrared light but practically no ultraviolet.

Small quartz tubes containing 1 cc of oxalated plasma or of fibrinogen solution were exposed to the influence of the rays for 10, 20 and 30 minutes. Use of a fan prevented temperature variations exceeding 3°C. The total energy reaching the tubes was practically the same for all experiments and amounted to 200 micro-cal. cm²/min.

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[1] Pincussen, L., *Photobiologie*, Leipzig, 1930.

[2] Spiegel-Adolf, M., *Ergeb. der Physiol.* (Asher-Spiro), 1928, **27**, 382.

[3] Mond, R., *Arch. ges. Physiol.* (Pflueger), 1922, **196**, 540; 1923, **200**, 374.

[4] Howell, W. H., *Arch. internat. de Physiol.*, 1921, **18**, 269.

[5] Hektoen, L., and Welker, W. H., *J. Infect. Dis.*, 1927, **40**, 706.

For testing the coagulation time we proceeded as follows:

(1) *Plasma.* To the tubes containing the irradiated specimens and the nonirradiated control an excess of $CaCl_2$ solution was added for eliminating the oxalate and activating the prothrombase, and the coagulation time tested in a water-bath at 37°C.

(2) *Fibrinogen.* The same quantity of a suspension of dry thrombase[6] in water was added to the irradiated specimens and to the control and the coagulation time tested at 37°C.

In the graphs the results are recorded. Fig. 1 deals with the effect on fibrinogen, Fig. 2 with the effect on plasma in 2% NaCl solution. The abscissa gives the time of irradiation, the ordinate the

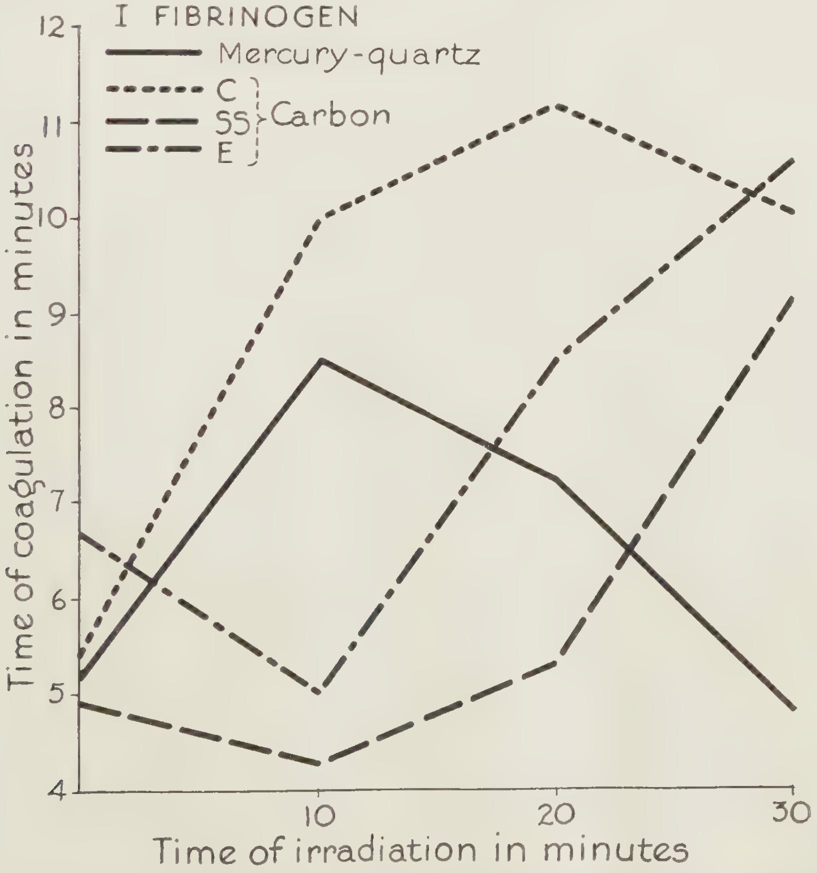

FIG. 1.

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[6] Mellanby, J., *Proc. Roy. Soc. B.*, 1933, **113**, 93.

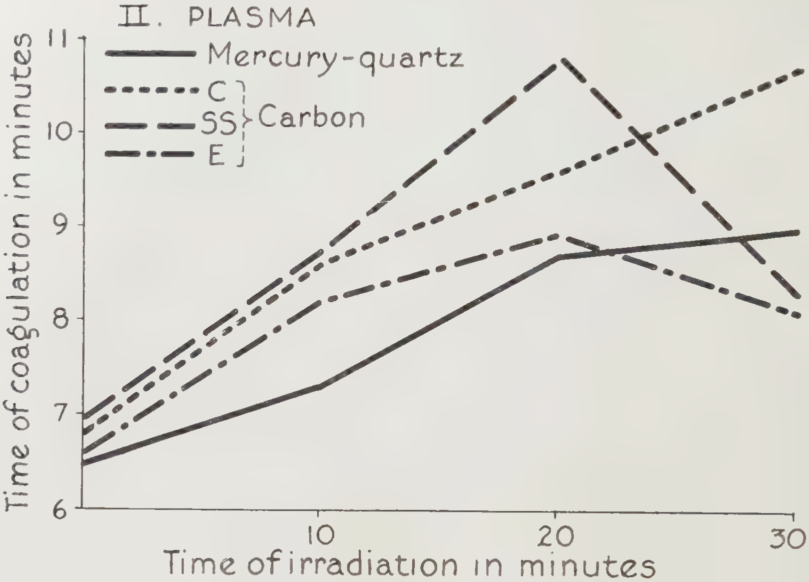

FIG. 2.

time of coagulation, both in minutes. Each curve in the graphs shows the average of 12-15 experiments.

From these graphs 2 groups seem to emerge: the first including the light sources with the strong U. V. output, the second including those with little or no U. V. and a highly effective visible and infra-red spectrum. This is true for the coagulation time of fibrinogen solutions and of plasma, although the trend of these two groups is different.

For the strong U. V. emitters, mercury-quartz light and C-carbon light, the coagulation time of the fibrinogen solution goes up after being irradiated and goes down again when the irradiation lasted longer than 10 or 20 minutes. For plasma, there is an upward trend too, but there is no reverse action after longer exposure to the light; the longer the irradiation lasts, the more coagulation is delayed.

After irradiation with light sources containing less U. V. and more visible and infra-red rays (Carbon SS and E) the coagulation time of fibrinogen is decreased when the solutions were exposed to the rays for 10 minutes, whereas longer irradiation shows a very distinct trend toward delayed coagulation. For plasma there is after an irradiation for 10-20 minutes an increase of the coagulation time followed by a reverse behavior after prolonged exposure to the rays.

For explaining these results we have, besides the differences in the applied radiation, to consider the differences in physico-chemical and chemical composition of the irradiated solutions. In both cases, we deal with colloids. The differences are: (1) the fibrinogen solutions are supposed to contain practically only fibrinogen whereas the plasma contains all of the other proteins of the blood. (2) The fibrinogen solutions are colorless while the plasma shows a yellowish color. (3) The plasma contains the enzyme prothrombase which like other enzymes[1] may be affected by the ultraviolet rays.

To account for the effect of the rays on fibrinogen it seems probable that the differences in coagulation-time are at least partially due to changes in the colloidal behavior which, if the strong U. V. emitters were used, are more or less reversible. The same may be true for short time irradiations with the light sources SS and E which contain mostly visible and infra-red wave-lengths, whereas in the second phase an irreversible change takes place, probably due to an alteration in the molecule itself effected by the heat-rays.

In the plasma the 3 factors mentioned above combine in their effect. The color of the liquid delays the effect of the U. V. whereas on the other hand more visible and infra-red rays are absorbed. Some of the rays are taken up by the proteins other than fibrinogen; thus, in all the effect of the rays on the plasma is expected to be smaller than on fibrinogen-solutions, and the curves in Graph 2 are running much smoother than those for fibrinogen coagulation. Nevertheless the action on the colloid persists and we explain the curves for E and SS by changes in the colloidal behavior.

For the strong U. V. emitters (mercury-quartz and C-carbon) these effects are obviously overpowered by the action of the short waves on the enzyme prothrombase. In spite of the protection given the enzyme by the colloids present in the plasma the activity of the prothrombase is lowered and even annihilated by U. V., the same as observed for all enzymes tested until now.

10995 P

Preparation and Assay of Inhibitor of Gastric Secretion and Motility from Normal Human Urine.

H. NECHELES, MARTIN E. HANKE* AND ERICH FANTL.

From the Department of Gastro-Intestinal Research, Michael Reese Hospital, and the Department of Biochemistry, University of Chicago.

Necheles and Lim[1] prepared biodialysates from the blood of dogs, which produced an increase of pancreatic and gastric secretion. In some experiments, however, inhibition of gastric secretion was observed. The latter finding was not followed up until reports of a number of hormones in the urine of man and animal prompted one of us (H.N.) to search for the presence of a gastric inhibitory principle in human urine.[2]

Lim, *et al.*,[3] described the extraction of a gastric inhibitory substance from intestines which they called enterogastrone. They found small amounts of this substance in other tissues and in a sample of Ivy's cholecystokinin. Walawski[4] described a substance with similar properties extracted from the large intestine. Lim's findings were confirmed by Ivy's laboratory, and they (and Sandweiss) confirmed our findings of an enterogastrone-like substance in human urine.[5]

Chemical Methods. Normal human urine is treated with 1% of $(NH_4)_2SO_4$, and the gelatinous precipitate (which forms in part even without $(NH_4)_2SO_4$), is washed twice with 1.5% $(NH_4)_2SO_4$, and then stirred vigorously with about 1 volume of water and 4 volumes of 95% alcohol. Thus there is obtained a 65% alcohol infusion, equal to about 1.5% of the volume of the original urine. The solid is collected by centrifugation, and the supernatant treated with one volume of 95% alcohol which causes the formation of a copious white precipitate equivalent to about 15 mg per liter of urine. Both solids are progressively fractionated by (1) extracting with water, (2) adding 2.1 volumes of 95% alcohol

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* This work was supported by a grant from the Rockefeller Foundation.

[1] Necheles, H., and Lim, R. K. S., *Chin. J. Physiol.*, 1928, **2**, 415; Necheles, H., *Chin. J. Physiol.*, 1927, **1**, 69.

[2] Necheles, H., communications to Dr. D. J. Sandweiss and Dr. A. C. Ivy, 1937; *Am. J. Digest. Dis.*, 1938, **5**, 471; *Internat. Physiol. Congr.*, Zurich, 1938.

[3] Kosaka, T., and Lim, R. K. S., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 890; Lim, R. K. S., *Quart. J. Exp. Physiol.*, 1933, **23**, 263.

[4] Walawski, J., *Med. Doswiadcz. i. Spot.*, **11**, 1930, 348.

[5] Gray, J. S., Bradley, Wm. B., and Ivy, A. C., *Am. J. Physiol.*, 1937, **463**, 118; Gray, J. S., Wieczorowski, E., and Ivy, A. C., *Science*, 1939, **89**, 489.

and 0.2% NaCl to get a 65% alcohol precipitate. (3) adding an equal volume of alcohol to the supernatant fluid to get an 80% alcohol precipitate. These fractionations are repeated according to a definite plan, until at the ninth reprecipitation 2 water-soluble fractions are obtained, one completely precipitated by 65% alcohol and the other entirely soluble at 65%, but precipitated at 80% alcohol. Both give strong protein tests, and can be precipitated from aqueous solution by ammonium sulfate (45% of saturated), picric or tannic acid.

Biological Assay. The substances were assayed for toxicity, and for effects on motility and secretion. Crude preparations increased secretion of bile and contracted the gall bladder. Purer preparations did not affect the gallbladder, blood pressure, respiration and secretion of saliva, urine or bile. Gastric secretion was assayed on Pavlov dogs following a standard meat meal, and motility on gastrostomized dogs following injection of insulin. These procedures were chosen after many others had been tried and found unsatisfactory. With 0.5 mg injected intravenously, the volume of secretion was decreased from 0.6 to 0.3 cc per minute, and the acidity from 100 free and 130 total, to 60 and 105 clinical units, respectively. With 1 mg the volume was changed from 1.0 to 0.1 cc per minute, and the acidity from 130 free and 150 total, to 0 and 25 clinical units respectively. On motility 2 mg caused a slight inhibition for 1 hour, while 4 mg caused complete inhibition for more than 3 hours. Secretion is inhibited by a smaller dose than is motility.

The fact that a large number of preparations show the same relative effect on secretion and motility, indicates that the same chemical substance inhibits both functions. Our substance may be identical with Lim's enterogastrone; however, the inhibition of gastric motility and secretion with various peptones[6] leaves this question open. Refractoriness to our preparations as described by others[7] was not observed. Small doses of our substance do not inhibit the stomach, when given *per os.* Boiling for 2 minutes destroys about 50% of the activity.

Summary. Method of extraction and assay of a substance from normal human urine are described. The substance is non-toxic and depresses or abolishes gastric secretion and motility in doses of 1-4 mg. The same chemical substance seems to inhibit both functions.

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[6] Brunemeier, E. H., and Carlson, A. J., *Am. J. Physiol.*, 1914-15, **36**, 191; Dadley, J., and Koskowski, W., *C. R. Soc. Biol.*, Paris, 1932, **109**, 1028; Thomas, J. E., and Crider, J. O., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 825.

[7] Gray, J. S., and Wieczorowski, E., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 324.

10996 P

Gas-Gangrene-Toxin Production.

G. B. Reed, J. H. Orr and Mary C. Baker. (Introduced by R. G. Sinclair.)

From Queen's University, Kingston, Canada.

Since Bull's original demonstration that *Cl. welchii* is toxigenic it has been customary to cultivate this and other gas-gangrene anaërobes for toxin-production in chopped-meat media, rich infusion-broths or serum-broths. It seems evident from the literature that many workers have found these methods to give highly variable yields of toxin. In our experience different batches of such media made in an apparently similar manner, inoculated with the same strain of organism and handled in as nearly as possible an identical manner, give very different yields of toxin. The recent experience with the production of diphtheria toxin has indicated that more consistent yields may be obtained by substituting for complex infusions, media of known, or for the most part known, chemical composition.

After several preliminary trials the following medium was found to give fairly high yields of toxin which are consistent from batch to batch with all the toxin-producing members of the gas-gangrene group of organisms:

NaCl	2.	g
$MgSO_4$ $7H_2O$	0.02	,,
Na_2HPO_4 $12H_2O$	5.76	,,
KH_2PO_4	0.24	,,
Gelatin, Difco	50.	,,
Peptone, Bacto	10.	,,
Glucose	2.	,,
Water	1000	cc

pH 7.7, Autoclaved at 15 lb.

The most consistent results have been obtained with freshly prepared and autoclaved media. While small inocula give good growths, more uniform toxin-yields have followed the use of inocula consisting of 3% of a 5 to 6 hour culture in the same medium.

Many modifications of this medium have been tried, particularly various proportions of gelatin and peptone. Variations of 1 to 10% of gelatin and 0.1 to 10% of peptone give fair yields of toxin but with a maximum at about 5% gelatin and 1% of peptone. Bacto peptone is slightly superior to several others tested. Fair yields of toxin are obtained with 5% of gelatin and no peptone. Larger concentrations of glucose and other sugars, fermentable by the organism in question, give somewhat more rapid growth but the yield of toxin is conspicuously lower probably due to the greater acid-production. Variation in the phosphate-content has relatively little influence provided the buffer value is sufficient to prevent a fall in pH from the initial 7.7 to not less than 6.2 to 6.0 in 18 to 24 hours.

Relative viscosity and formol titration indicate that a rapid hydrolysis of the gelatin begins 1 to 2 hours after inoculation and continues at a rapid rate beyond the time toxin production is at a maximum, 18 to 24 hours. *Cl. welchii* cultures, in this medium, transform 16 to 18% of the gelatin-nitrogen to amino-nitrogen by the time maximal toxin-production is reached, whereas cultures of *Cl. septicum* at the time of maximal toxin-production show slightly less than half this amount of amino-nitrogen.

Measurable amounts of toxin are produced after 6 to 8 hours' incubation, the concentration increases conspicuously up to about 18 hours, shows relatively little change for the next 4 to 6 hours, then gradually decreases until it practically disappears after 2 to 3 days' incubation of the culture. The toxin may be filtered through Seitz or sintered glass (Jena G5 on 3) filters with comparatively slight loss of toxicity, compared with centrifuged cultures, provided the first 20 to 30 cc of filtrate, in the case of small filters, is discarded.

Toxins from this medium may be satisfactorily precipitated with ammonium sulphate and dried. Good toxoids are made by a relatively short period of formolization.

Various toxigenic strains of the 3 principal species of gas-gangrene organisms grown in this medium produce the following concentrations of toxin, estimated as hæmotoxin. Hæmotoxin-titration endpoint was taken as the dilution of toxin that will hæmolyze 50% of a 2% suspension of rabbit's red cells:

Cl. welchii	1-1000 to 1-4000
Cl. septicum	1-100 to 1-200
Cl. novyi	1-100 to 1-200

10997

Insulin Sensitivity of Monkeys after Section of the Hypophyseal Stalk.*

J. R. Brobeck, H. W. Magoun and S. W. Ranson.
From the Institute of Neurology, Northwestern University Medical School.

Disturbances of carbohydrate metabolism including hypersensitivity to insulin have been described in animals following the production of hypothalamic lesions (Ingram and Barris;[1] Cleveland and Davis;[2] Ingram and Winter;[3] Brobeck[4]), and it has been suggested that such disturbances may be due to interruption of nervous connections with the anterior lobe of the hypophysis (Davis, Cleveland and Ingram;[5] Houssay[6]).

The possibility that the fibers of the hypophyseal stalk are concerned with this function has been disproved by administering insulin intravenously to monkeys (*Macaca mulatta*) which had fully recovered from section of the infundibular stalk just proximal to the dorsal surface of the gland.

The details of operation and exact locations of the lesions have been reported (Magoun and Ranson[7]). The procedure for the tests was the same as that already reported for cats (Brobeck[4]) except that the dosage of insulin used for the monkeys was 0.1 unit per 0.2 kg body weight, and blood samples were taken before insulin injection and afterwards at the end of each hour for 3 hours. Blood sugars were estimated by the Randles and Grigg[8] modification of the Folin-Wu method.

The results of tests on 3 normal monkeys are compared with tests on 4 operated animals in Graphs I and II. The responses of the 2 groups of animals were quite similar, and the operated animals appeared no more sensitive to insulin than normal. Recovery from hypoglycemia did not take place as rapidly in the monkeys as it does in normal cats (Brobeck[4]), but there was no difference in the amount

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* Aided by a grant from the Rockefeller Foundation.

[1] Ingram, W. R., and Barris, R. W., *Am. J. Physiol.*, 1936, **114**, 562.

[2] Cleveland, D., and Davis, L., *Brain*, 1936, **59**, 459.

[3] Ingram, W. R., and Winter, C. A., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 449.

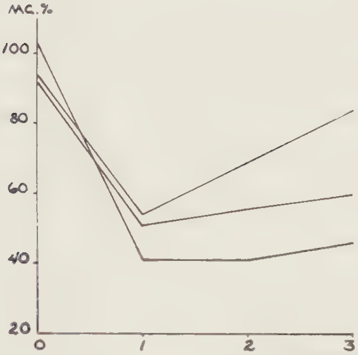
[4] Brobeck, J. R., *J. Lab. Clin. Med.*, 1939, in press.

[5] Davis, L., Cleveland, D. A., and Ingram, W. R., *Arch. Neurol. and Psychiat.*, 1935, **33**, 592.

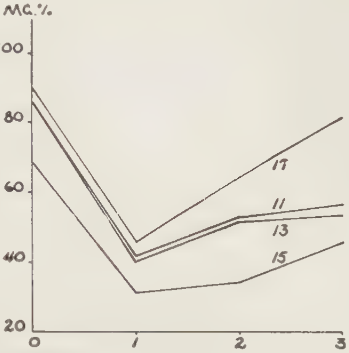
[6] Houssay, B. A., *New England J. Med.*, 1936, **214**, 971.

[7] Magoun, H. W., and Ranson, S. W., *Anat. Rec.*, 1939, **75**, 107.

[8] Randles, F. S., and Grigg, W. K., *J. A. M. A.*, 1924, **82**, 684.



Graph 1.
Insulin responses of normal monkeys.



Graph 2.
Insulin responses of monkeys 11, 13, 15, and 17, after section of the hypophyseal stalk.

of recovery shown by normal and operated monkeys, and no external hypoglycemic symptoms were observed.

Fatal hypoglycemia occurred, however, in one insulin experiment not included on either graph; this animal was monkey 16 in which the stalk section was comparable to the others of the series, but in which there was also extensive degeneration of the anterior lobe. The degeneration appeared to be the result of anemic softening and was probably due to interruption of the vessels described by Wislocki and King[9] in the region of the severed stalk. There was no evidence of hemorrhage and the autopsy revealed no other unusual pathology. Smaller areas of similar degeneration were present in the anterior lobes of 2 of the animals which reacted normally to insulin (monkeys 13 and 15), but the anterior lobes of monkeys 11 and 17 appeared to be intact.

By means of a planimeter, estimations were made of the volumes of normal anterior lobe tissue present after the hypophyses of these animals had been fixed, sectioned, stained and mounted: in monkeys 11 and 17 the glands appeared normal with volumes of 15.3 and 13.5 cu mm respectively; in monkeys 13 and 15 in which small areas of degeneration were present the volumes of normal anterior lobe tissue were respectively 8.8 and 6.2 cu mm; in monkey 16 which died in hypoglycemia the volume of the normal tissue was 4.1 cu mm. The volume of anterior lobe tissue from 4 normal monkeys was respectively 10.9, 14.5, 15.6 and 17.0 cu mm.

Insulin hypersensitivity following hypophysectomy of the monkey has been reported by Hartman, Firor and Geiling[10] and by Smith, Tyndale, Dotti and Engle[11] who also found that partial hypophysectomy did not produce the marked increase in sensitivity which followed complete removal of the gland. The present experiments indicate that insulin responses may be normal when only half of the anterior lobe is left intact.

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[9] Wislocki, G. B., and King, L. S., *Am. J. Anat.*, 1936, **58**, 421.

Infundibular connections between the hypothalamus and pars distalis, however, are not essential for normal insulin sensitivity in the monkey, and are therefore probably not essential for normal carbohydrate metabolism.

10998

Assay of Progesterone by Intrauterine Application in the Rabbit.

ARTHUR L. HASKINS, JR. (Introduced by George W. Corner.)

From the Department of Anatomy, the University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

The crop gland test for the lactogenic hormone as performed by Lyons and Page,[1] the local feather test for estrone by Greenwood and Blyth,[2] and the intravaginal test for estrogenic substance by Yerby,[3] serve as examples of the efficacy of the local application of a hormone for analytical purposes. The application of progesterone to the uterus has been shown by McGinty, Anderson and McCullough[4] to be a more sensitive test for its presence than introducing the hormone into the blood stream.

Previous biological assay or test procedures for the presence of progesterone have involved the subcutaneous or intramuscular injection of the hormone over a period of 5 days, removal of the uterus on the sixth day and subsequent histological examination of the uterus. The criterion of the activity of the hormone of the corpus luteum was the degree of progestational proliferation in evidence in the uterus. The test for progesterone as described by Corner and Allen[5] utilized as test animals bilaterally oöphorectomized mature rabbits. The degree of proliferation in the uterus was expressed by them in terms of + to ++++ reactions, + reactions being those of least progestational proliferation.

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[10] Hartman, C. G., Firor, W. M., and Geiling, E. M. K., *Am. J. Physiol.*, 1930, **95**, 662.

[11] Smith, P. E., Dotti, L. B., Tyndale, H. H., and Engle, E. T., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 247.

[1] Lyons, W. R., and Page, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1049.

[2] Greenwood, A. W., and Blyth, J. S., *Quart. J. Exp. Physiol.*, 1935, **25**, 267.

[3] Yerby, D., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 496.

[4] McGinty, D. A., Anderson, L. P., and McCullough, H. B., *Endocrinol.*, 1939, **24**, 829.

Clauberg[6] and later McPhail[7] modified this procedure to the extent that immature rabbits were used as test animals. These animals were primed with subcutaneous injections of estrone for a period of days preceding the intramuscular injection of progesterone. Immature rabbits as treated by Clauberg and McPhail were found to be more sensitive to the presence of progesterone, since divided daily injections totalling 0.5 mg brought about a uterine proliferation equal to injections totalling approximately 1 mg in the mature rabbit.

McGinty and his collaborators found that single intrauterine doses of progesterone in amounts of 5 to 0.5 gamma caused a local proliferation of ++ to +++ which is equal to that proliferation caused by the injection of 0.5 mg of progesterone in the McPhail test. Applications of amounts of progesterone from 0.5 to 0.125 gamma in all cases produced a definite proliferation. However the decreasing intensity of the reaction indicates that these small doses are approaching the minimum required for a recognizable progestational action to occur within the uterus.

The following experiments were carried out to test the results as reported by McGinty, Anderson and McCullough.

Immature female rabbits, average weight 1239 g, were treated with 150 international units of amniotin over a period of 6 days. On the seventh day they were anesthetized with ether, the abdomen was opened and the uterus exposed. A segment of each horn of the uterus was isolated by two ligatures approximately 3 cm apart; care was taken to leave an adequate blood supply to the segment. A third ligature was applied loosely between the others and close to the lower ligature. The progesterone-containing solution to be applied was forced into the lumen of the isolated segment by means of a syringe inserted beneath the loose ligature, which was gradually tightened as the solution was injected and the needle withdrawn.

Crystalline progesterone* was applied in a 0.1 cc solution of peanut oil or lanolin in dilutions as indicated in Tables I and II. The

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[5] Corner, G. W., and Allen, W. M., *Am. J. Physiol.*, 1929, **88**, 326.

[6] Clauberg, C., *Zbl. f. Gynak.*, 1930, **54**, 2757.

[7] McPhail, M. K., *J. Physiol.*, 1934, **83**, 145.

* The crystalline progesterone used in this study was generously supplied by the Schering Corporation through Dr. Erwin Schwenk.

isolated segment in the opposite horn was injected with peanut oil, lanolin or blood serum from the non-pregnant guinea pig as required for a control.

Mature rabbits when used were bilaterally oöphorectomized at the time of the application of progesterone in addition to the above procedure. The mature rabbits were not primed with estrone. All animals were killed 72 hours following the application of progesterone, the injected segments were removed and examined histologically. The degree of reaction is expressed according to the McPhail scale.

Results. The control segments in all but 2 cases exhibited no trace of progestational proliferation. These 2 cases were found in the control horn after injection of 1 mg and 0.3 mg of progesterone into the opposite horn. This fact would seem to indicate that the action of locally applied progesterone remained local up to a maximum dose, at which point there was seepage into the blood stream.

The progesterone-injected segments of the immature rabbit uteri showed a positive reaction in all but one animal, this being one of those injected with 0.25 gamma, as is shown in Table I. Lanolin when used as an alternative solvent for the progesterone, produced results similar to that of the peanut oil solution as indicated in Table II. However in all cases the variability of the reaction as compared

TABLE I.

Rabbit wt, g	Progesterone in 0.1 cc peanut oil, gamma	Reaction	Control peanut oil, cc	Reaction
1245	1000	++++	.1	+ to ++
1245	300	++++	.1	+
1232	100	0 to +	.1	0
1264	25	0 to +	.1	0
1069	5	++	.1	0
1416	1	+	.1	0
1470	1	+	.1	0
1700	1	+	.1	0
1530	0.5	++	.1	0
1480	0.25	+	.1	0
1818	0.25	0	.1	0
736	0.25	+ to ++	.1	0
854	0.25	+ to ++	.1	0
1013	0.25	++	.1	0
3200*	100	0	.1	0
2900*	100	0	.1	0
2841*	1	0	.1	0
2554*	0.5	0	.1	0
2841*	0.5	0	.1	0
2727*	0.25	0	.1	0
3009*	0.25	0	.1	0

* Bilateral oöphorectomy at time of application of progesterone, mature rabbits.

TABLE II.

Rabbit wt, g	Progesterone in 0.1 cc Lanolin, gamma	Reaction	Control Lanolin, cc	Reaction
963	10	+ to ++	.1	0
1135	1	+	.1	0
1226	1	+ to ++	.1	0

TABLE III.

Rabbit wt, g	Pregnancy serum, cc	Reaction	Control non-pregnancy serum, cc	Reaction
1192	.4*	++ to +++	—	—
1084	.2	+ to ++	.2	0
1205	.2	+ to ++	.2	0
1432	.1	0	.1	0

* Ether-soluble contents of 0.4 cc of pregnancy serum, dissolved in 0.2 cc of peanut oil.

to the amount of progesterone administered indicates that this test as it stands would be impractical as a quantitative test.

Mature rabbits were used with the thought that they might exhibit a more predictable or constant reaction. However amounts of progesterone from .25 to 100 gamma failed to cause a recognizable proliferation of the mature uterine endometrium.

In addition to a corroboration of the results of McGinty, Anderson and McCullough, it was found that the immature rabbits to which blood serum from the pregnant guinea pig was applied by intrauterine injection (Table III) exhibited a + to ++ reaction with application of 0.2 cc of serum. A stronger reaction occurred in the uterus to which the ether-soluble portion of 0.4 cc of pregnancy serum, dissolved in 0.2 cc of peanut oil, was applied. The uterus of the rabbit to which 0.1 cc of pregnancy serum was applied showed no reaction. The pregnancy serum was obtained from 2 guinea pigs, 2 weeks after mating.

Blood serum from the non-pregnant guinea pig was injected for control purposes. The control segments in all cases exhibited no recognizable progestational activity.

A number of factors may be involved in the variability of reaction encountered with the intrauterine application of progesterone. If the total proliferation produced by a given amount of progesterone were always equal, a slight variability in the size of the segment isolated would alter the apparent progestational proliferation as encountered in a single histological section of the whole segment. The size or volume of the isolated segment may vary because of differences in the calibre of the segment and the bore of its lumen. The length of the segment isolated is of course subject to experimental error. The reaction to progesterone may also vary according to differences in the blood supply of the isolated segment. Finally the reaction to progesterone in the individual may be subject to individual variation caused by the interaction of other endocrine secretions.

It is apparent, however, from McGinty's work that the local application of progesterone is superior as a qualitative test to the previous methods of intramuscular injection because of its relative sensitivity.

10999

Indoluria and Its Relation to Sulfur Deficiency.

DANIEL H. BASINSKI AND GEORGE T. LEWIS
From the Department of Biochemistry, Emory University, Ga.

Indole is a product of the action of bacteria on protein. Whether this action takes place in the gastrointestinal tract or in the body proper the organism is faced with the problem of the disposal of the resulting indole. Normally, as far as can be determined by analysis of excretions, the indole is oxidized to indoxyl, this in turn conjugated with potassium hydrogen sulfate and the indican thus formed is excreted in the urine. It is conceivable that if oxidation is deficient, if the liver fails in its function of conjugation, or if the supply of sulfate is deficient, free indole may appear in the urine.

Indoluria has been reported by various investigators, Cantelli,[1] Schour and Rosengarten,[2] Forbes and Neale[3] but Vaughan[4] failed to observe it. It has also been shown by Vaughan[5] and Carnes and Lewis[6] that the urine contains some compound which is readily acted upon by *E. coli* to produce indole.

If the indoluria is due to a lack of sulfate to effect the conjugation, the sulfur deficiency should be manifested elsewhere in the body. It is known that practically all the urinary sulfate enters the organism as unoxidized sulfur and hence, it is not necessary to find an actual sulfate deficiency in the excretions to prove the point. Lightbody and Lewis[7] as well as Mitchell and associates[8] have shown that when the sulfur supply is insufficient in the rat, the sulfur and cystine content of the hair is lowered. Presumably if the same condition should occur in the human, it would extend to the fingernail, another epidermal tissue whose cystine content has recently been repeatedly determined. If, therefore, free indole appears in the urine as the result of sulfur deficiency, the fingernails might be expected to reflect the condition in a lowered cystine content.

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[1] Cantelli, O., *Riforma Medica*, 1922, **38**, 481, abstract in *J. Am. Med. Assn.*, 1922, **79**, 508.

[2] Schour, M., and Rosengarten, C., *Klin. Wochschr.*, 1930, **9**, 1751, 1968.

[3] Forbes, J. C., and Neale, R. C., *J. Lab. Clin. Med.*, 1934-35, **20**, 1017.

[4] Vaughan, S. L., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 623.

[5] Vaughan, S. L., *J. Lab. Clin. Med.*, 1937, **22**, 399.

[6] Carnes, H. E., and Lewis, G. T., *J. Lab. Clin. Med.*, 1938, **23**, 459.

It seemed to us worth while to determine whether indoluria and low fingernail cystine co-exist in the same individual. Since qualitative tests for free indole can be carried out fairly rapidly in quantity, it was decided to attack the problem from this angle first and then to determine fingernail cystine in those cases which showed a positive test.

A number of difficulties were encountered in the establishment of a sensitive and reliable qualitative test for indole in the urine. False positives might result from (1) bacterial contamination before or after voiding, (2) decomposition of indole precursors during manipulation of the specimen, (3) the presence of compounds which give color reactions similar to those given by indole. In order to rule out any possible bacterial activity after the voiding of the sample, all positive indole tests were checked on freshly voided specimens. In this way the only danger of encountering indole produced subsequent to the excretion of the urine was in cases of G.-U. tract infection. Only one such case (No. 19) is known to have been present in our series.

We have felt that possibly the method used by Forbes and Neale[3] in their studies of indoluria might give rise to false positive tests because of the extensive manipulations involved. For this reason as well as for the saving of time in carrying out an extended series we turned to the method of Vaughan.[4] After rather extensive trial we adopted the following modified procedure.

Two cc portions of redistilled petroleum ether are pipetted into 6 inch test tubes graduated at 7 cc. Urine is poured in up to the mark, the tube tightly stoppered with a clean cork stopper and the specimen extracted by inverting the tube 50 times. Usually a slight emulsification results but this can be quickly dispelled with a drop or 2 of 95% ethyl alcohol. One cc of the petroleum ether layer is transferred to a small agglutination tube and 0.5 cc of Ehrlich's reagent (2 g p-dimethylaminobenzaldehyde in 100 cc 20% HCl) added. If indole is present a red color results on shaking.

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[7] Lightbody, H. D., and Lewis, H. B., *J. Biol. Chem.*, 1929. **82**, 485, 663.

[8] Beadles, J. R., Braman, W. W., and Mitchell, H. H., *J. Biol. Chem.*, 1930, **88**, 623; Smuts, D. B., Mitchell, H. H., and Hamilton, T. S., *J. Biol. Chem.*, 1932, **95**, 283.

If this presumptive test is positive it must be repeated after the removal of as many interfering substances as possible. To this end an excess (usually 3 cc) of saturated lead acetate solution is added to 5 cc of a freshly voided urine specimen. Filter and to 2 cc of the filtrate add 1 cc of Ehrlich's reagent. The precipitate of lead chloride which forms does not interfere with the observation of the pink color. The color can be extracted with amyl alcohol if desired.

Urobilinogen very readily gives a color with Ehrlich's reagent (Wallace and Diamond[9]) but is precipitated by the lead acetate. Skatole and indole acetic acid which might perhaps be expected to arise by the same reaction which gives rise to indole, give a color with the reagent but only when relatively much larger amounts are present and in addition the color is distinctly different in quality and easily distinguishable from the pink given by indole. Indole gives a distinct color in a dilution of 1:1,000,000. It should be pointed out, however, that the p-dimethylaminobenzaldehyde must be of the highest purity if false positive reactions are to be avoided. Our failure to realize this forced the discarding of several weeks' work at the beginning of our study.

We felt, therefore, that we had a procedure for the qualitative detection of indole in urine which was delicate and quite specific.

Using this procedure we have examined urine samples from 275 hospital patients from the colored medical ward of Grady Hospital. The patients have in practically all cases been males. From this number 22 patients have shown indoluria. Table I contains the clinical diagnoses for these individuals. The total is not that of all the patients whose urines were examined because clinical conditions which never showed an indoluria are not represented.

Fingernail clippings were taken from those patients who showed a positive test for indole. The clippings were brought to the laboratory and soaked for several hours or overnight in 70% alcohol. This served to soften any dirt and cuticle adherent to the fragments of nail and these were then removed by scraping with a dull scalpel. The clean clippings were dried in an electric oven at 100°C overnight.

Fifty to 60 mg portions of the clean, dry nail parings were weighed out and placed in 5 cc of 20% HCl. Hydrolysis was effected by immersion of the flask in an oil bath maintained at 120°-125°C for

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[9] Wallace, G. B., and Diamond, J. S., *Arch. Internal Med.*, 1925, **35**, 698.

TABLE I.

Diagnosis	No. of patients	Indoluries	% cystine in fingernails
Pneumonia	58	6	(9.0-10.7)*
Heart disease	54	6	(10.3-11.4)*
Pneumonia and heart disease	3	1	8.4
Pulmonary tuberculosis	13	2	11.2, 9.7
Cerebral hemorrhage	3	3	9.4, 9.7, 10.2
Diabetes mellitus	17	1†	10.5
Pyelo-nephritis	1	1	10.4
Typhoid fever	8	1	11.4
Bronchiectasis	1	1	8.5
Total	158	22	**10.0 avg**

* Parentheses indicate range of values observed.

† This single positive case from the diabetic series was in diabetic coma when the specimen was obtained.

6 hours. Cystine was determined in the hydrolysate by the Rossouw and Wilken-Jorden modification[10] of the Sullivan method. Duplicate analyses were read against different standards and the determinations were repeated until agreement within 0.2% of cystine was obtained.

As will be seen from Table I, the cystine values varied between 8.4% and 11.4% with an average of 10%. The cystine content of fingernail clippings from presumably normal individuals of the same race and approximately the same age as determined by this method in our laboratory ranged from 7.9% to 11.7% with an average of 9.9%.

It is evident from these data that indoluria as we have encountered it is not accompanied by a lowered content of cystine in the fingernails. And, if the level of cystine in the fingernails be accepted as an index of the available supply of sulfur in the body, the explanation of the presence of free indole in the urine does not appear to be failure of conjugation because of lack of available sulfur.

Summary. Free indole was found in 22 of 275 specimens of urine obtained from hospital patients. Fingernail clippings from the patients having indoluria had a normal cystine content. It seems improbable that indoluria is due to failure of conjugation because of lack of available sulfur.

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[10] Rossouw, S. D., and Wilken-Jorden, T. J., *Onderstepoort J. Vt. Sci. and An. Indus.*, 1934, **2**, 361.

11000 P

Diabetes in Rats and its Alleviation with Sodium Chloride.*

James M. Orten and Henry B. Devlin

From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit.

The occurrence of "diabetes" as indicated by a low tolerance to glucose in approximately 60% of adult rats of the "Yale" strain, whereas Wistar rats showed a normal tolerance, has been reported[1]. The incidence of the low glucose tolerance became progressively less in younger animals, all rats 50 days of age or less showing a normal tolerance. Some dysfunction of the anterior pituitary was suggested as a possible cause of the impaired carbohydrate metabolism.[2]

Similar observations have been made in this laboratory and the work has been extended in the following way.

Adult male and female albino rats of the "Yale" strain, (Connecticut Agricultural Experiment Station strain) were fasted 16 to 18 hours and were then administered intraperitoneally 350 mg glucose per 100 g body weight, as a sterile 8.75% solution. At the intervals shown in the table, blood was drawn from a tail vein and its sugar content was promptly determined by the Somogyi micro-modification of the Shaffer-Hartmann method.[3] The tolerance to glucose was likewise determined on a group of partially pancreatectomized adult rats.

The results, summarized in Table I, demonstrate clearly that approximately 40% of the intact animals had a low tolerance† to glucose, similar to that shown by the partially pancreatectomized rats. The blood sugar level rose to excessively high values and remained high during the 5-hour period of observation. It should be pointed out, however, that the response of an individual intact rat was not invariably the same. As was also observed by Cole and Harned[1], changes in the type of tolerance curve from diabetic to normal and vice versa on repeated tests were not uncommon. In a few studies, the data of which are not included in this paper, rats of the Wistar

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* Aided by a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

[1] Cole, V. V., and Harned, B. K., *Endocrinology*, 1938, **23**, 318 .

[2] Harned, B. K., and Cole, V. V., *J. Biol. Chem.*, 1939, **128**, xxxix.

[3] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

† Some investigators[1] have arbitrarily designated a blood sugar value of 180 mg % at the end of the 5-hour period as the upper limit for a "normal tolerance." This value has also been adopted in the present study.

strain and 50-day-old rats of the Yale strain invariably showed normal tolerances to glucose.

The data obtained on the "low-tolerance" group of intact rats reveals an interesting trend in the blood sugar values. Usually, there was a sharp rise at the 30-minute interval, followed by a decrease at the 60- and 90-minute periods, then a second rise, frequently exceeding the first even at the end of the 5-hour period of observation. Since it is known that the injection of a hypertonic solution of glucose into the peritoneal cavity causes a migration of sodium and chloride ions into the peritoneal fluid[4], it appeared possible that a reduction of plasma sodium, or perhaps an altered ratio of plasma sodium to potassium, might be involved in the second rise in the blood sugar values and thus in the low tolerance. Support for such a possibility is found in the observations that sodium chloride administration increases the deposition of glucose in the liver as glycogen[5] and improves the utilization of glucose by human diabetics,[6] and that the administration of potassium salts to rats produces an hyperglycemia[7].

For these reasons, the effect of the intraperitoneal injection of glucose solution containing sodium chloride was studied in the rats previously used. A 1.58% concentration of sodium chloride, isotonic to the 8.75% glucose solution, was employed.

It is evident from a comparison of these data (Table I,B) with

TABLE I.
Average Blood Sugar Values of Intact and Pancreatectomized Rats Administered Glucose* with and without Sodium Chloride by Intraperitoneal Injection.

Group	No. of rats	"True" Blood Sugar—Mg %						
			Minutes after glucose administration					
		Fasting	30	60	90	120	180	300
A. Glucose Alone.								
1. Normal tolerance, intact rats	17	79	211	202	184	158	158	127
2. Low tolerance, intact rats	10	75	268	243	231	224	257	295
3. Partially pancreatectomized rats	8	88	309	318	322	320	337	307
B. Glucose with Sodium Chloride.								
1. Normal tolerance, intact rats	5	83	251	214	162	150	128	100
2. Low tolerance, intact rats	6	74	276	248	220	194	154	119
3. Partially pancreatectomized rats	4	90	319	240	176	155	121	104

* 350 mg C. P. Glucose per 100 g body weight.

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[4] Darrow, D. C., and Yannet, H., *J. Clin. Invest.*, 1935, **14**, 266.

[5] Crabtree, D. G., and Longwell, B. B., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 705.

[6] McQuarrie, I., Thompson, W. H., and Anderson, J. A., *J. Nutrition*, 1936, **11**, 77.

[7] Silvette, H., Britton, S. W., and Kline, R., *Am. J. Physiol.*, 1938, **122**, 524.

those obtained on the rats given glucose alone that the response of the normal tolerance intact rats to injected glucose was not significantly altered by sodium chloride. On the other hand, the administration of *sodium chloride with glucose* to the "low tolerance" rats resulted in a normal utilization of glucose in all instances. The partially pancreatectomized rats likewise showed a normal tolerance to injected glucose solution containing sodium chloride.

As yet, no explanation of the favorable effect of sodium chloride on the utilization of intraperitoneally administered glucose by the intact diabetic rat and the partially pancreatectomized rat can be advanced. Further studies on this problem are in progress.

Summary. The report that approximately one-half of adult rats of the "Yale" strain show a diabetic tendency, as indicated by a low tolerance to glucose administered intraperitoneally, has been confirmed. This tendency is more marked in older rats than in younger ones. The administration of sodium chloride isotonic with the injected glucose results in a normal tolerance to glucose, both in intact rats showing the diabetic trait and in partially pancreatectomized rats.

11001

Ultrafiltration of the Virus of Infectious Avian Encephalomyelitis.

Peter K. Olitsky and Johannes H. Bauer

From the Rockefeller Institute for Medical Research, and the Laboratories of the International Health Division of the Rockefeller Foundation, New York.

The causal agent of the recently described[1-3] infectious avian encephalomyelitis or "epidemic tremor of young chickens" has been shown to pass through Berkefeld V and N, and Seitz 1- and 2-pad filters.[1-3] The present paper concerns experiments on ultrafiltration of the virus through gradocol membranes; thus a more precise measurement of its size might be acquired.

The membranes employed had an effective filtration-area of 5 cm^2 and were prepared after the method of Elford,[4] as modified in certain

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[1] Jones, E. E., *J. Exp. Med.*, 1934, **59**, 781.

[2] Van Roekel, H., Bullis, K. L., and Clarke, M. K., *J. Am. Vet. Med. Assn.*, 1938, **93** (N.S. **46**), 372.

[3] Olitsky, P. K., *J. Exp. Med.*, 1939, **70**, in press.

[4] Elford, W. J., The sizes of viruses and bacteriophages, and methods for their determination, in *Handbuch der Virusforschung*, edited by Doerr, R., and Hallauer, C., Vienna, Julius Springer, 1938, Vol. 1, pp. 126-181.

particulars by Bauer and Hughes.[5] The procedure of filtration followed that employed by the latter:[6] positive nitrogen-pressure of 76 cm Hg and 9 or 10 cm^3 of either stock-filtrate, or supernate after centrifugation, of the virus were used with each membrane. The stock materials just mentioned were prepared as follows: The filtrate was obtained by spinning at 2500 rpm for 10 minutes in a horizontal centrifuge a 1:10 dilution in 25% broth in saline solution of 3 pooled brains removed from birds at the height of experimental avian encephalomyelitis.* The resulting clear fluid was then passed through a 1-pad Seitz filter. The supernate was prepared by spinning the clear fluid just described in the Bauer-Pickels open-air centrifuge[7] at 12,000 rpm for 1 hour; 0.1 cm^3 of each of the filtrates, or of the control materials as indicated in the table, was inoculated intracerebrally* in 9- to 16-day old chicks. When signs of encephalomyelitis were apparent their specific nature was ascertained by the familiar, characteristic pathological changes,[3] by transfer of the tissues of central nervous system to fresh birds, or by both methods (Table I).

The tabulated results indicate that with the exception of Experiment 3 in which, it is assumed, defective membranes may have been used, there is an apparent limit of filtration through a membrane of a porosity of 73 mμ A.P.D. While this is not as sharply demarcated as that obtained with certain other viruses such as with the two strains

TABLE I.
Ultrafiltration Experiments with Avian Encephalomyelitic Virus.

Avg pore diam. of membrane in mμ	Results of inoculation of filtrates in chicken			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
150	5/6*	—†	—	—
125	6/6	—	—	—
100	3/6	—	—	—
83	—	1/6	1/6	—
73	5/6	0/6	1/6	2/8
60	0/6	0/6	3/6	0/8
50	0/6	0/6	1/6	0/8
40	0/6	0/6	—	0/8
Virus control:				
Unfiltered	4/4	6/6	6/6	6/6
Seitz filtrate	2/4	4/6	—	—
Supernate after centrifugation 1 hr at 12,000 rpm	—	—	5/6	6/6

* Numerator represents the number of chickens showing encephalomyelitis and denominator the number of chickens used in the test.

† Not tested.

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[5] Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

[6] Bauer, J. H., and Hughes, T. P., *Am. J. Hyg.*, 1935, **21**, 101.

* Full ether anesthesia was employed for all operative procedures on birds.

[7] Bauer, J. H., and Pickels, E. G., *J. Bact.*, 1936, **31**, 53.

of equine encephalomyelitic virus,[8] which are characterized by (a) high concentration of virus per unit-volume, and (b) a uniform, and readily discernible specific reaction of inoculated animals, two factors regarded by Elford[4] as essential for clear-cut results, yet the end-point of the avian virus that has neither of these high potencies,[3] is sufficiently indicated in the present tests.

In accordance with the Elford definition of end-point, *viz.,* a membrane just able to retain completely all the dispersed particles,[4] the limiting membrane in this instance is the one of A.P.D. of 60 mμ. Hence, by applying the Elford formula,[4] the size of the viral particle is in the range of 20 to 30 mμ. It is of iterest that this size is within the same range as that demonstrated for the viruses of equine encephalomyelitis and St. Louis encephalitis.[9]

Conclusion. The virus-particles of infectious avian encephalomyelitis as present in suspension of brain of chickens affected by the experimental disease were found to have a diameter of 20 to 30 mμ as determined by ultrafiltration through gradocol membranes.

11002

L Type Variant Forms in Cultures of Various Bacteria.*†

L. Dienes

From the Department of Pathology and Bacteriology of the Massachusetts General Hospital, Boston, and the Robert W. Lovett Memorial for the Study of Crippling Disease, Harvard Medical School, Boston.

In a recent publication[1] data were presented supporting the conclusion that the L1 organism isolated by Klieneberger[2] from cultures of *Streptobacillus monilliformis* is a variant form of the bacillus and not a symbiant. Dawson and Hobby[3] came to the same conclusion after studying a streptobacillus strain isolated from a patient suffering from rat bite fever.

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[8] Bauer, J. H., Cox, H. R., and Olitsky, P. K., Proc. Soc. Exp. Biol. and Med., 1935, **33**, 378; Tang, F. F., Elford, W. J., and Galloway, I. A., *Brit. J. Exp. Path.*, 1937, **18**, 769.

[9] Bauer, J. H., Fite, G. L., and Webster, L. T., Proc. Soc. Exp. Biol. and Med., 1934, **31**, 696; Elford, W. J., and Perdrau, J. R., *J. Path. and Bact.*, 1935, **40**, 143.

* The expenses of this investigation were defrayed in part by a grant from the Commonwealth Fund.

† This is Publication No. 38 of the Robert W. Lovett Memorial.

[1] Dienes, L., *J. Inf. Diseases*, 1939, **65**, 24.

[2] Klieneberger, E., *J. Path. and Bact.*, 1935, **40**, 93; 1936, **42**, 581.

[3] Dawson, M. H., and Hobby, G., Third International Congress for Microbiology, Abstracts of Communications, 1939, New York.

During the past year plates inoculated with routine bacteriological specimens have been examined for the presence of colonies having the characteristics of L type[1]. All suspected colonies were stained and examined *in situ* on the agar. The technic of making such examinations has been described[1]. Employing this method L types of colonies were observed in the cultures of 4 unrelated Gram negative bacteria.

An abundant characteristic growth of L type colonies was observed in the cultures of a Gram negative bacillus obtained repeatedly and in pure culture from a dog bite wound. A transient phlegmon developed following the bite but the etiological significance of the bacillus remains doubtful since similar bacilli are often encountered as saprophytes. The patient did not present symptoms of systemic invasion. Culturally, the bacillus is similar to the yellow Gram negative saprophytic strains (genus Flavobacterium). Growth on blood agar plate is distinctly yellow. Freshly isolated strains did not grow on Endos medium. Lactose was not fermented. Acidity but no gas was produced in dextrose and maltose.

Two types of growth were observed on blood agar plates. In thickly seeded areas the growth was abundant and uniform and consisted of regularly shaped small bacilli. For a few millimeters surrounding the thickly seeded areas small colonies were visible. Around large single colonies the distribution of small colonies resembled the satellite growth of influenza bacilli. Transplants from both the large and small colonies grew in a similar way and strains with different properties could not be isolated from the culture. While the large colonies consisted of regular shaped bacilli, the bacteria in the small colonies after a few hours' growth became pleomorphic, some swelling to form large oval bodies. At the same time tiny curved filaments began to grow into the agar. After 24 hours tiny secondary colonies similar to young L colonies were visible in the agar below the small colonies. The growing edge of this tiny colony consisted of very fine curved filaments. The older parts of the growth were transformed into deeply stained granules and fairly large round bodies. After 48 hours, the central part of the colony did not stain well and the whole resembled a loose colony of tiny diphtheroids. Unlike L1 colonies, they did not produce a rim of large round bodies on the surface of the agar. It might be pointed out, however, that this rim does not develop in the L1 colonies if they grow under a streptobacillus colony. Under the large colonies consisting of regular shaped bacteria, no L type colonies developed.

All efforts to isolate this L variant in pure culture have been unsuccessful. For this reason its properties, such as filtrability and the possibility of reversion to regular bacillary forms could not be examined.

Tiny secondary colonies similar in appearance to the L type colonies also developed under colonies of influenza bacilli. A strain cultured from a wound produced such colonies to a marked degree. They were seen also in smaller numbers in cultures of a strain isolated from spinal fluid. Thus far the L type of growth has not been observed in cultures from the throat. The wound strain was very pleomorphic on blood agar plates and many bacteria swelled into large round bodies. After 24 hours the edges of the colonies were surrounded with tiny secondary L type of colonies invading the medium. Small secondary colonies consisting of typical influenza bacilli may also appear on certain media under the colonies. They are, however, very different in appearance from the L type of colony. The latter consist of elements smaller than regular bacteria, are loosely constructed and grow by extension of fine, curved filaments.

In blood broth cultures of the wound strain many bacteria were transformed into large round bodies. Six to 8 hours after planting such cultures on blood agar plates the fine curved filaments of developing L colonies appeared to grow out from these large bodies. This was observed repeatedly in those instances where the large bodies were isolated on the surface of the medium. This observation suggests that the L type of growth originates from the large bodies. The chance of observing this process directly is very slight because only a few of the large bodies develop into L type of colony.

The tiny deep colonies produced by the yellow strain and by influenza bacilli resemble the L1 colonies of *Streptobacillus monilliformis* in the following points: The appearance of the colonies and the morphology of these organisms are very similar and yet very different from their parent strains. The colonies consist of very small elements. They become embedded and develop in the medium. Their development is preceded by pleomorphism and swelling of the bacteria so that they appear as large round bodies. It would therefore seem probable that the small secondary colonies in the cultures of all these strains represent analogous formations.

L type colonies were observed to develop, although in a less pronounced form, under the colonies of a strain of *Bacillus funduliformis*. This strain was isolated from the blood and from an infected joint of an individual who subsequently died. The bacilli at the edge of the colonies were pleomorphic and produced large swollen forms. Under the colonies many tiny secondary colonies developed in the agar medium. Most of these consisted of regular shaped bacteria. However, a few presented the typical morphology of L type colonies. Certain strains of *Streptobacillus monilliformis* behave similarly in that they produce tiny secondary bacillus colonies together with very few L colonies.

L type colonies were observed also in the cultures of several strains of the colon group. The characteristic type of growth of these strains will be reported in a subsequent note[4]. When these strains were isolated they produced many large spherical or spindle shaped

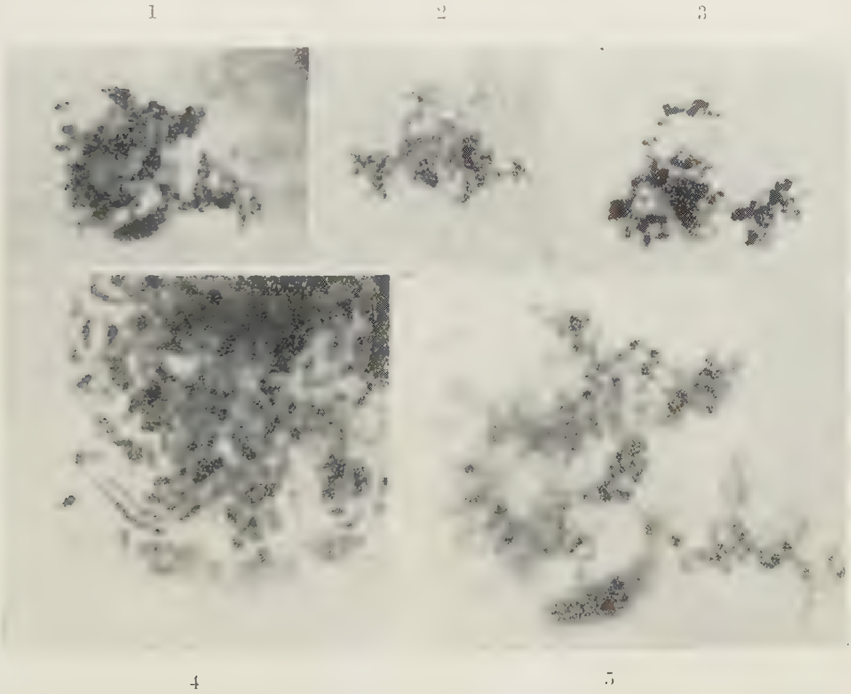

FIG. 1.

The photomicrographs were made from the colonies of the yellow Gram negative bacillus (genus *Flavobacterium*). The colonies were stained *in situ* with toluidine blue. The thin strips of agar media containing the colonies were then transferred to slides and dried.

Photographs 1 and 2 represent tiny L type colonies. The magnification (1:1000) is insufficient to show the constituent elements of the colony but gives a general impression of their appearance.

Photographs 3 and 5 represent similar colonies with higher magnification (1:2000) and show somewhat better the constituent elements. Photograph 5 is an enlargement of photograph 1.

Photograph 4 shows a small bacterium colony from the same preparation. Note the pleomorphism of bacteria and the presence of large swollen bodies.

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[4] Dienes, L., PROC. SOC. EXP. BIOL. AND MED., in press.

forms. Adjoining these large forms one observes frequently a growth of very fine curved filaments into the agar medium and the presence of very small, lose L type of colony. This type of colony develops only on blood agar plates and has never been observed on plain, dextrose, ascitic or boiled blood agar. Compared with the L type colonies of *Streptobacillus monilliformis* or those of the yellow bacillus, these L colonies remained very small and only rarely was a compact colony observed. In agar cultures stained *in situ* with the methods used in this study, groups of stain granules are seen occasionally under leucocytes or other cells. If methylene blue or azure is used the color of the precipitate is reddish while the young L type colonies are stained bright blue. Attention is called to this stain precipitate to avoid confusion in those cases in which the L type colonies remain small.

It would appear therefore that the formation of L type colonies is not an exclusive property of the *Streptobacillus monilliformis*. It might be suggested that with the use of appropriate technic such variant forms might be observed more frequently.

Summary: Cultures of various bacteria on blood agar plates were stained *in situ* by methods previously described. Examination of cultures of a Gram negative bacillus (of the genus *Flavobacterium*) so stained revealed tiny secondary colonies similar in appearance and morphology to the L1 variant of the *Streptobacillus monilliformis*. The same phenomenon was observed, in a less pronounced degree, in cultures of *Bacillus influenzae, Bacillus fundulliformis* and in cultures of certain strains of colon bacilli.

11003

Heterophile Antibodies Developed During Prophylactic Rabies Immunization.

Irene Rosenfeld and H. E. McDaniels
(Introduced by Lloyd Arnold)

From the Laboratories of Mt. Sinai Hospital, Illinois Department of Public Health, and Department of Bacteriology and Public Health, University of Illinois College of Medicine, Chicago.

Aside from producing resistance to rabic infection, the injection of relatively large amounts of rabbit-brain suspension may be expected to induce the formation of various antibodies. A type of generalized reaction clinically resembling infectious mononucleosis is occasionally encountered in patients undergoing antirabic immunization. In such patients, a simple test for heterophilic antibodies, using only sheep-erythrocytes, is difficult to interpret unless one knows what effect the injections of rabbit-brain suspensions have upon heterophilic agglutinin-titers.

Davidsohn[1–2] reported hemolysins and agglutinins for sheep's red blood cells in the blood of patients treated with normal or with immune horse serum. Paul and Bunnell[3] demonstrated an increase of heterophilic antibodies in the blood of patients suffering from infectious mononucleosis. Davidsohn and Walker[4] differentiated the antibodies present in serum disease from those present in infectious mononucleosis by absorption of the specimen of serum with guinea pig's kidney and of another sample of the same serum with rabbit's kidney. They consider the antibodies in infectious mononucleosis to be heterophilic in nature, but not of the Forssman heterophilic type. They defined heterophilic antibodies as "antibodies that have the ability to react with antigens which are apparently entirely unrelated to those that stimulated their production." Bailey and Raffel[5] concluded that the heterophilic antibodies in infectious mononucleosis are not the Forssman type because they were not absorbed by a known carrier of Forssman antigen, namely, guinea pig's kidney; though horse kidney, another carrier of Forssman antigen, had some ability to absorb the heterophilic antibodies. Stuart, Tallman, and Brintzenhoff[6] reported an increase in rabbit-cell agglutinins in the majority of patients with horse-serum-sickness.

The purpose of our present investigation was to study the antibody-responses in prophylactic rabies-immunization and to compare the nature of antibodies in serum-sickness, infectious mononucleosis, and normals with those found in the serum of patients who received rabies-vaccine. Fifty-nine patients were treated with Semple rabies-vaccine. Blood was obtained* before treatment and 10 to 15 days after treatment. The specimens were tested for the presence of agglutinins for the red cells of sheep, rabbit, and guinea pig. The technic of absorption and the titrations were carried out according to the methods described by Davidsohn[7].

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[1] Davidsohn, I., *J. Immunol.*, 1929, **16**, 259.

[2] Davidsohn, I., *J. Immunol.*, 1930, **18**, 31.

[3] Paul, J. R., and Bunnell, W. W., *Am. J. Med. Sci.*, 1932, **183**, 90.

[4] Davidsohn, I., and Walker, P., *J. Clinical Path.*, 1935, **5**, 455.

[5] Bailey, J. H., and Raffel, S., *J. Clin. Invest.*, 1935, **14**, 228.

[6] Stuart, C. A., Tallman, J., and Brintzenhoff, E., *J. Immunol.*, 1935, **28**, 85.

* We are indebted to the Chicago Board of Health for collection of the blood specimens and other excellent cooperation.

[7] Davidsohn, I., *J. Am. Med. Assn.*, 1937, **108**, 289.

TABLE I.
Erythrocyte-Agglutinins Before and After Rabies-Immunization. Average Titers in 59 Patients.

Sheep		Rabbit		Guinea pig	
before	after	before	after	before	after
1:4	1:24	1:62	1:258	1:12	1:44

The injections of rabies-vaccine stimulated the formation of antibodies against the erythrocytes of sheep, rabbit, and guinea pig. There is a more definite and consistent increase in the agglutinative titer for the red cells of guinea pig and rabbit than for those of sheep. In this respect the antibodies formed during rabies-immunization differ from those present in infectious mononucleosis and serum-sickness. In these diseases there is a consistent increase in the sheep-cell agglutinins, regardless of the increase for rabbit and guinea pig cells.

Absorptions were carried whenever the titer for sheep cells was 1:28 or higher. In all cases the following agents were used: guinea pig and rabbit kidney, beef-erythrocytes, and kaolin. Titers of the sheep-cell agglutinins were determined before and after absorptions. The effect of absorption was expressed in percentages of antibodies removed. If the antibodies were left the same, the percentage of absorption equals zero; if they were removed completely, the absorption equals 100%. Table II gives the absorbing effect of these agents upon sera with titers of 1:28 or higher.

The readiness with which all antigens removed the agglutinins for sheep red cells suggests that the antibodies developed during rabies-immunization are of the heterophilic type. The kidney of the guinea pig and the erythrocytes of the sheep, both carriers of the Forssman antigen, removed completely the agglutinins for red cells of the sheep. However, erythrocytes of beef and kidney of the rabbit, known to lack Forssman antigen, also completely removed the agglutinins for the sheep cells. Therefore, the results of absorption indicate that

TABLE II.
Effect of Absorption.

No. of cases	Titer before absorption	Percentages of absorption of agglutinins for the red blood cells of sheep with				
		Kidney of		Erythrocytes of		
		Guinea pig	Rabbit	Beef	Sheep	Kaolin
16	1:28	100	100	100	100	63
4	1:56	100	100	100	100	50
2	1:112	100	100	100	100	50

the antibodies developed during rabies-immunization are heterophilic in nature but do not belong to the special Forssman group.

Summary. Heterophilic antibodies developed during rabies-immunization differ from the heterophilic antibody developed in true serum sickness and from the heterophilic antibodies present in cases of infectious mononucleosis.

11004

Correlation between Movements of Gravid Uterus and Formation of Histamine.

H. HANDOVSKY. (Introduced by C. Heymans.)

From the Department of Pharmacology, University of Ghent, Belgium.

Harding and Fort[1] first noted the presence of a considerable amount of histamine in the placenta. This has been confirmed several times since. As an accumulation of histamine could be dangerous for normal pregnancy it seemed consistent to investigate whether histaminase is present in the placenta. Danforth and Corham[2] found histaminase to be present in the human placenta though in extremely variable amounts. Danforth[3] demonstrated that the amount of histaminase to be found in the placenta "shows some correlation with the efficiency of uterus contractions of women during labor."

During researches—Daels and Donatelli[4-6]—on the motility of the cat's uterus (about 150 animals) we had the opportunity to examine the motility of the uterus of 7 pregnant cats. These observations seem to us to be in correlation with the problem mentioned above and this is why we want to publish them. The cats in our experiments were narcotized with chloralosane; the distal end of one uterus horn was fixed by a thread which was connected by a Jackson tube to a level which registrated the movements. The abdomen was closed, the animal warmed.

Table 1 shows the results of these 7 experiments.

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[1] Harding, V. J., and Fort, C. A., *J. Biol. Chem.*, 1918, **35**, 29.

[2] Danforth, D. N., and Corham, F., *Am. J. Physiol.*, 1937, **119**, 394.

[3] Danforth, D. N., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 319.

[4] Handovsky, H., and Daels, J., *Internat. Congress of Physiology*, 1938, Kongressbericht II, p. 209.

[5] Handovsky, H., and Daels, J., *C. r. Soc. Biol.*, 1939, **131**, 150.

[6] Daels, J., *Arch. internat. Pharmacodyn.*, in press.

TABLE I.

No.	Length of foetus, cm	Period of pregnancy*	Uterus movements	Blood pressure during contraction of the uterus
16	10.0	46 to 57 days	Every 2 min one movement lasting 1 min (see Fig. 1)	No change
28	2.3	< 14 days	,,	,, ,,
30	2.2	,,	,,	,, ,,
77	3.4	,,	,,	,, ,,
56	3.2	,,	,,	Short fall of pressure
62	5.2	15 days	,,	,, ,, ,, ,,
141	4.5	14 to 15 days	,,	,, ,, ,, ,, (see Fig. 1)

* Calculated according to Gros, G., *Le cycle génital de la chatte,* Thèse, Université d'Alger, 1936, p. 110.

While pregnant uteri of other species hardly show any movements[7] the uteri of our 7 pregnant cats showed movements of similar kind: every 2 minutes there arises one movement, which lasts about 1 minute. (Fig. 1).

In 3 animals out of the 7 the general blood pressure dropped for about half a minute (Fig. 1) at the moment when the uterus started contracting. We never observed that phenomenon in the uteri of non-pregnant animals. We cannot explain why all the 7 gravid

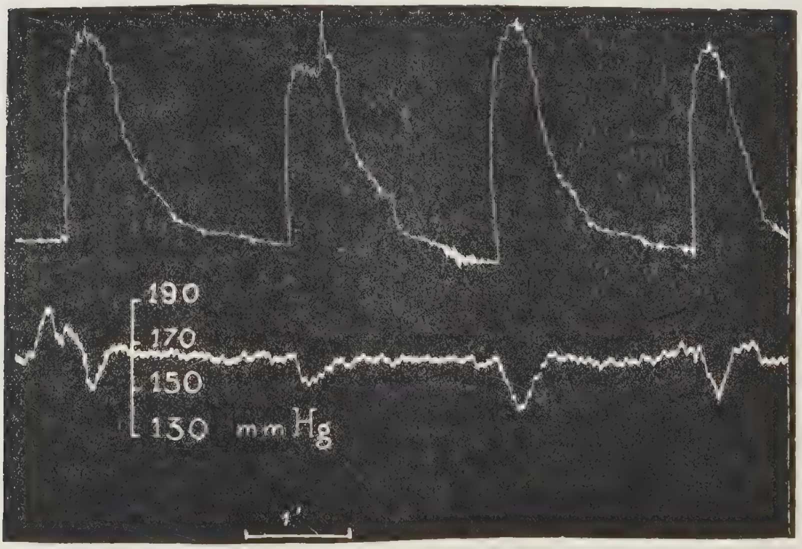

FIG. 1.
Cat 141. (Table I. Uterine movements. Blood pressure. Time.)

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[7] *Cf.* Reynolds, S. R. M., *Physiology of the Uterus,* New York, 1939, p. 1.

uteri showed movements of the same type in spite of the different periods of pregnancy and why in 3 cases only the fall of blood pressure occurred.

We suggest that inside of the pregnant uterus (in the placenta) of the cat a rhythmic production of histamine occurs, which provokes the contractions of the uterus, and that a certain amount of histamine may escape the destruction by the histaminase, come into the general circulation and cause the short, but distinct fall of blood pressure. This hypothesis would agree with the hypothesis of Danforth mentioned above.

11005

Further Observations of Phenothiazine in Experimental Trichinosis.*

JAMES B. MCNAUGHT, RODNEY R. BEARD AND FLOYD DEEDS.

From the Departments of Pathology, and Public Health and Preventive Medicine, and the Bureau of Agricultural Chemistry and Engineering,† U. S. Department of Agriculture, at the Department of Pharmacology, Stanford University School of Medicine, San Francisco, California.

A variety of therapeutic agents have been tried in experimental *Trichinella spiralis* infestations with but limited success.[1] A recent preliminary experiment indicated that 6 weeks' continuous use of a diet containing 0.25% phenothiazine reduced the severity of trichinous infection in rats 74%.[2] Further observations of the actions of this drug have been made and are herewith reported.

Methods. Adult white rats of the Wistar strain were used throughout. The general methods of drug administration, infection and muscle larvae counts were the same as before[2] except as noted in the individual experiments. Each experiment ran for 6 weeks. The results were evaluated by calculating the standard error of the mean for the larvae counts of the 4 animals in each group, and by calculating "t", according to Fisher's methods.[3] Variations with a chance

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* Supported in part by the Rockefeller Fluid Research Fund of the Stanford University School of Medicine.

† Food Research Division Contribution No. 464.

[1] van Someren, V. D., *Brit. Med. J.*, 1939, **1**, 376.

[2] McNaught, J. B., Beard, R. R., and DeEds, F., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 17.

[3] Fisher, R. A., *Statistical Methods for Research Workers*, 1934, 5th Ed., Oliver and Boyd, Edinburgh.

probability greater than 1 in 100, as indicated by the "t" table, were not considered significant.

Results. Notable, though incomplete, protection with phenothiazine had been previously reported[2] and it is here considered as Experiment 1 (Table I). *Experiment 2* was performed in order to ascertain the time when the administration of phenothiazine was most effective and whether a larger dose of the drug would further diminish the muscle-infestation.

Twenty-four female rats were segregated into 6 groups of 4 each. Each rat was infected with a quantity of rat meat containing 1535 encysted larvae. One group on the standard rat diet served as controls. The remaining 5 groups were placed, during consecutive weeks, on the same diet containing 0.5% phenothiazine. When these groups were not receiving medicated diet, they were on control diet. The results obtained and other pertinent details are presented in Table I. The total number of larvae recovered from each of the treated groups surpassed the number obtained from the control group. This result, namely that double the dose of phenothiazine given the first, second, third or fourth weeks, or continuously for 6 weeks, after infestation caused no reduction in muscle larvae seemed incompatible with our previous results.[2] However, the experiments differed in the following respects: (1) The concentration of phenothiazine in the diet of the animals of Experiment 2 was double that of Experiment 1; (2) The animals of Experiment 1 received the drug for 2 days prior to infection, while medication began after infection in Experiment 2; (3) The rats of Experiment 1 were infected by doses of 2350 encysted larvae while those of Experiment 2 received only 1535; (4) Older and heavier animals, showing a 20% gain in weight in 6 weeks, were used in Experiment 1, while the younger animals of Experiment 2 averaged a gain of 49%, except for those receiving continuous medication, which showed only a 20% gain; (5) Due to a temporary shortage of animals at the time of Experiment 1, male rats were used for the controls and female rats for phenothiazine administration. Only females were used in Experiment 2. Experiment 3 was designed to aid in clarifying the apparent discrepancy.

Experiment 3. Twenty-four rats of practically the same weight were segregated into 6 groups of 4 each. One group of males and one group of females served as controls and 4 groups of females received medication either before or after infection as shown in Table I. It is seen that, when female controls and female test animals were used, there was no protective action from phenothiazine, as in Experiment 2. The final yield of larvae in animals receiving pheno-

TABLE I.

Phenothiazine in Experimental Trichinosis of Rats.

Exp. No.	Group* No.	Sex	Initial Group wt, g	Gain in wt, %	Treatment with Phenothiazine	Total drug intake, g	No. of larvæ		Intake-Output Ratio
							Given	Recovered	
1	1 (C)	M	797	20			2350	762,400	1:81
	2	F	633	18	Prophylactic† and curative‡	3.78	2350	198,720	1:21
2	1 (C)	F	428	54			1535	804,100	1:131
	2	F	424	37	1st week only	1.03	1535	1,146,600	1:186
	3	F	440	52	2nd ,, ,,	.96	1535	900,900	1:146
	4	F	448	51	3rd ,, ,,	1.46	1535	842,100	1:137
	5	F	440	53	4th ,, ,,	1.17	1535	985,200	1:160
	6	F	424	21	6 weeks continuously	6.38	1535	1,105,200	1:180
3	1 (C)	F	743	2			1700	311,000	1:46
	2 (C)	M	739	20			1700	1,112,800	1:164
	3	F	735	4	Prophylactic	.42	1700	299,000	1:44
	4	F	733	0	,,	1.27	1700	249,700	1:37
	5	F	746	—4	,, and curative	3.86	1700	638,900	1:94
	6	F	736	—4	Curative	6.71	1700	553,120	1:81

* Four rats in each group.

† Drug was administered prior to infection.

‡ ,, ,, ,, after infection.

C—Controls.

thiazine prophylactically (previous to and concurrent with infection) in concentrations of 0.25% (Group 3) and 0.5% (Group 4) was not significantly lowered. Prophylaxis and continuous therapy with a phenothiazine concentration of 0.25% (Group 5), and continuous use of 0.5% phenothiazine in the diet after infection (Group 6), doubled the yield of larvae. The females were older and heavier than those used previously so that the gain in weight by the controls and those receiving light prophylaxis was only 2 to 4%, while the heavier medicated rats lost 4% in weight. That this mild weight loss probably was not due to the heavier infestation is indicated by the fact that the male controls containing twice the number of muscle larvae gained 20% during the same period. The males were not as old as the females but were comparable in weight. It has been shown previously[4] that the continuous administration of phenothiazine in the heavier dosage range caused some toxicity in rats as indicated by diminished rate of growth. It may well be that this toxicity lowered the resistance of the animals to the parasitic infestation.

In comparing the male controls of Group 2 with the female test animals, it was found that there was an apparent reduction in infestation of from 48 to 77%. These conditions also held in Experiment 1, where there was a reduction of 74%. Clearly, the apparent reduction in the 2 experiments was due to the fact that the females had much lighter infestations than the males fed the same number of larvae.

These results show conclusively that phenothiazine is ineffective in experimental trichinosis. The carefully controlled experiments made indicate a new field for study of the differences in yield of muscle larvae in male and female rats of various ages when infected with similar doses of *T. spiralis.* Campbell[5] recently reported that female rats infected by eggs of *Taenia taeniaformis* yielded only 67% as many liver cysts as did the males. Our observations indicate a similar situation in Trichinella infections.

Conclusions. Contrary to our previously published observations, no evidence was found that phenothiazine administered prior to, concurrent with, or after infestation, has any appreciable value in experimental trichinosis in rats. There is strong evidence of a sex variation in the susceptibility of rats to Trichinella infection, females having higher resistance as indicated by the number of encysted Trichinella found after feeding standard doses of larvae.

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[4] Eddy, C. W., Cox, A. J., and DeEds, F., *J. Ind. Hyg. and Toxic.*, 1937, **19**, 574.

[5] Campbell, D. H., *Science*, 1939, **89**, 415.

11006

Temperature Reactions in Mice Infected with Pneumococci.*

W. P. LARSON, RAYMOND N. BIETER, MILTON LEVINE AND W. F. MCLIMANS.

From the Departments of Bacteriology and Pharmacology, University of Minnesota.

White and Parker[1] have recently demonstrated that concentrations of sulfanilamide which had merely a bacteriostatic action on the streptococcus *in vitro* at temperatures below 39°C were bacteriocidal at 40°C. Since it is of importance to apply such findings to *in vivo* studies, a number of experiments were undertaken to determine the normal temperature of mice, the reason for variation if any existed, and lastly, the effect of temperature changes on the course of the pneumococcus infection in normal mice and those treated with sulfanilamide.

Walther[2] and Rolly and Meltzer[3] found that rabbits kept in a room warm enough to raise their body temperatures showed an increased resistance to pneumococcus infections. Loewy and Richter[4] raised the body temperature of rabbits by the Sachs-Aronson operation, or "heat puncture", and found that such animals were more resistant to pneumococcus infections than normal animals. Experiments on white mice have been reported by Ritzmann,[5] who found that mice kept for a period of weeks at a temperature of 95°F were less resistant to infections of streptococci and tetanus spores, and by Colvin and Mills,[6] who found that white mice kept at high temperatures were less resistant to streptococcus infections than animals kept at room temperature. McDowell[7] found that rats kept at a temperature of 83°F were more resistant to pneumococcus infections than rats kept at medium temperatures.

Temperature readings in our own experiments were made with a thermocouple attached to a Leeds and Northrup type K potentiometer. The warm junction was designed to penetrate the skin, and was inserted subcutaneously in making the temperature readings. The

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* Aided by a grant from the Graduate Medical Research Fund.

[1] White, H. J., and Parker, J. M., *J. Bact.*, 1938, **36**, 481.

[2] Walther, P., *Arch. f. Hyg.*, 1891, **12**, 329.

[3] Rolly, F., and Meltzer, *Dtsch. Arch. f. Klin. Med.*, 1908, **94**, 335.

[4] Loewy, A., and Richter, P. F., *Arch. f. path. Anat. und Phys.*, 1896, **145**, 49.

[5] Ritzmann, O., *Arch. f. Hyg.*, 1907, **61**, 355.

[6] Colvin, J. W., and Mills, C. A., *Science*, 1939, **90**, 275.

[7] McDowell, C., *Am. J. Hyg.*, 1923, **3**, 521.

average temperature of 126 mice in a series of 268 readings taken at different times was 100.6°F, with a standard deviation of 1.1°.

In order to raise the temperature of the animals, they were placed in a chamber maintained at 98°F. It was found that mice placed in such a room showed a rise in temperature of about 2° to 3° in less than ½ hour, and that the temperature remained in this range during the entire period they were kept in the chamber. Eighty-one readings on 17 mice kept at 98°F over a period of 3 days yielded an average temperature of 104.1°F with a standard deviation of ±0.95°F. Twenty-four normal mice remained under these conditions for 3 days without showing any signs of injury. The food intake of these animals was normal, although the water intake was higher than usual.

To study the effects of different external temperatures on the progress of Type II pneumococcus infections in mice several parallel experiments were conducted. Equal numbers of control and treated mice were kept (1) at room temperature (approximately 70°F) throughout the experiment, and (2) at 98°F for 3 days immediately following the pneumococcal inoculations. The experiments were conducted on equal groups of from 6 to 10 mice per group. A total of 50 mice was used in each group. The technic of drug administration was the same as that used previously.[8]

TABLE I.
The Sulfanilamide Intake for Each Group, in g per kg of Body Weight.

Day	At room temperature	At 98° F
1	.50	.49
2	.76	.76
Inoculation with pneumococcus Type II.		
3	.725	.81

TABLE II.
Time of Survival of Mice Treated with Sulfanilamide in Food and Inoculated Subcutaneously with 15,000 Lethal Doses of Pneumococcus Type II.

	Control Groups		Treated Groups	
	At room temperature	At 98° F	At room temperature	At 98° F
No. of mice	50	50	50	50
Survivors at 30 days	0	0	1	1
Range	18.5-52.5 hr	14-45.5 hr	20.5-252 hr*	20-60 hr
Mean	29.0 ”	20.6 ”	69.5 ”	30.1 ”
Standard error	1.08 ”	0.9 ”	5.7 ”	1.3 ”

* Figures for range, mean, and standard error in the treated groups are for those mice which died.

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[8] Bieter, R. N., Larson, W. P., Levine, M., and Cranston, E. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 202.

In an attempt to explain the apparent decrease in resistance of infected mice at 98°F, as compared to those kept at room temperature, readings were made on mice injected subcutaneously with the same dose of the pneumococcus as used above. These measurements were made on animals kept at room temperature after infection, and those kept at the higher temperature. In the former, the mice showed a steady decline in temperature, beginning about 14 hours after infection, and continuing until death. In some cases, the temperature immediately before death was as low as 84°F. At no time was there a rise in temperature above the normal range in any of the mice studied. In those mice kept at 98°F, the temperature remained above normal, and no drop was detected even in those examined shortly before death.

To eliminate the possibility of increased toxicity of sulfanilamide at the higher temperature, 24 mice were placed on the diet containing the same amount of drug used previously (0.5%) and were kept in the heated room. None of these mice showed any ill effects after 3 days, and the average intake of food was the same as in the animals infected with the pneumococcus.

In a recent communication from Perrin Long, we learn that he has measured temperatures of mice. His findings are in agreement with those here reported.

Summary. The body temperature of mice is markedly influenced by the surrounding temperature. Pneumococcic infections in mice are associated with a marked drop in temperature. Infected mice held at incubator temperature succumb more rapidly than those at room temperature. Incubator temperature promotes the infective processes in mice more than the protective action of sulfanilamide.

11007

Effect of Simultaneous Administration of Growth Complex and Estradiol on Mammary Gland of Hypophysectomized Rat.

IRA T. NATHANSON, DARREL T. SHAW AND CLIFFORD C. FRANSEEN.
(Introduced by Joseph C. Aub.)

From the Laboratories of the Collis P. Huntington Memorial Hospital, Harvard University.

In a study of the effect of sex hormones on the growth and development of the mammary gland of the rat, it was found by Reece, *et al.*, that estrogens were not capable of stimulating the gland in the absence of the hypophysis.[1] It was postulated by these investigators that the hormones acted upon the mammary gland by way of the pituitary gland. On the other hand, Astwood, *et al.*,[2] suggested that this failure to react to a known stimulus might be due in part to the nutritional deficiency which resulted as a consequence of hypophysectomy. The present experiment was undertaken therefore to ascertain whether the mammary gland could be stimulated in the hypophysectomized rat by the simultaneous administration of growth complex and estradiol benzoate. A possible effect from injection of estrogens on the rate of secretion of other pituitary hormones in intact animals is eliminated by the injection of a constant amount of growth complex in hypophysectomized animals.

A total of 70 stock rats was used in this experiment. Thirty-six of these were females, of which 22 were immature. Of the 34 males, 20 were immature. These were divided further into the subgroups which are outlined below. The immature animals weighed between 50 and 70 g. The mature weighed from 150 to 175 g.

Hypophysectomy on the animals which were subjected to the procedure was performed by the parapharyngeal route, using a technic previously described.[3] At the completion of the experiment the pituitary fossa of each of the animals was examined with a magnifying loupe to verify the fact that hypophysectomy had been complete. Daily weights were recorded on all animals during the course of the experiment. The gonads in both sexes showed complete atrophy at the termination of the experiment.

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[1] Reece, R. P., Turner, C. W., and Hill, R. T., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 204.

[2] Astwood, E. B., Geschickter, C. F., and Rausch, E. O., *Am. J. Anat.*, 1937, **61**, 379.

[3] Franseen, C. C., Brues, A. M., and Richards, R. L., *Endocrinology*, 1938, **23**, 292.

Estradiol benzoate was injected subcutaneously in daily doses of 1000 I.U. in 0.1 cc of sesame oil. The growth complex, after dilution with normal salt solution, was administered intraperitoneally daily in 2 divided doses equivalent to 1/50 cc of the extract (2 rat units).*

The hormones were given to the various animals in periods varying from 3 to 5 weeks. They were then sacrificed at intervals during the latter period when one could be reasonably certain that the changes resulting from hypophysectomy *per se* were complete. Whole mounts of the breasts were then made and examined.

Results: Group I—Control. Mature males, 2. Immature males, 2. Mature females, 3. Immature females, 3. The breast in this group showed typical development dependent upon the sex and stage of maturity.

Group II—Hypophysectomy Only. Mature males, 2. Immature males, 2. Mature females, 2. Immature females, 2. In this series the breast showed almost complete atrophy when the animals were sacrificed 3 to 4 weeks after hypophysectomy.

Group III—Hypophysectomy plus Growth Hormone. Mature males, 2. Immature males, 3. Mature females, 2. Immature females, 2. Injections of growth complex as outlined above were started 6 hours after hypophysectomy and continued for 3 to 4 weeks. The mature rats maintained their pre-treatment weight levels. The immature animals gained about 2 g per day until about the third week. Both groups after this period began to lose weight. Each animal was sacrificed as soon as weight loss became evident. The breasts of these animals showed almost complete atrophy.

Group IV—Estradiol Benzoate only. Mature males, 2. Immature males, 2. Mature females, 2. Immature females, 2. Injections of estradiol benzoate were given daily as outlined. All animals were sacrificed between 3 and 5 weeks after the onset of the experiment. Examination of the breasts revealed marked ductal and acinar proliferation and in some instances cyst formation.

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* We are indebted to Mr. Joseph Hutchinson of Ayerst, McKenna & Harrison for a generous supply of growth complex, and to Drs. Gregory Stagnell and Max Gilbert of the Schering Corporation for liberal quantities of estradiol benzoate. The growth complex, according to information received from the manufacturers, contained 100 Collip units of growth factor, 10 Collip units of adrenotropic factor, and 10 bird units of prolactin per cc. One growth unit is defined as that quantity which, when administered in two divided doses over a period of 10 to 15 days by intraperitoneal injection into recently hypophysectomized but otherwise untreated rats weighing between 80 to 120 g, causes an increase in body weight of one g per day. Our experiments check their findings in regard to growth hormone.

Group V—Hypophysectomy plus Estradiol Benzoate. Mature males, 2. Immature males, 2. Mature females, 3. Immature females, 3. Estradiol benzoate was injected daily commencing one day after operation. All animals were sacrificed between the third and fifth week. The breasts of all animals showed the usual atrophy, thus corroborating the work of the other investigators.

Group VI—Hypophysectomy plus simultaneous administration of growth complex and estradiol benzoate. Mature males, 4. Immature males, 7. Mature females, 4. Immature females, 9. Injections of growth complex were commenced 6 hours after operation, and continued daily thereafter along with estradiol benzoate in the usual dose. The injections were continued for 3 to 5 weeks. The animals were sacrificed at intervals during the last 2 weeks of the experiment. This group can be subdivided on the basis of weight change.

Subgroup A. Eight animals in which there was a definite gain in weight following operation. These were sacrificed at the first discernible sign of weight loss. Examination of the breasts in the group revealed a degree of development and activity which approached those of the untreated controls.

Subgroup B. Six animals,—no change from preoperative weight. The breasts in this group were intermediate between the normal controls and the atrophic changes seen after hypophysectomy alone.

Subgroup C. Ten animals—in which weight loss varied from 8 to 26 g from the preoperative level; the mature animals having the greatest weight loss. Examination of the breasts in this group revealed changes which varied in degree between those seen in subgroup B and the group with hypophysectomy only.

It would seem from the results given above that the nutrition of the animal is an important factor in determining the effect of estrogens upon the growth and development of the mammary gland in the rat. This is obvious since breast development and maintenance of the fully developed gland after simultaneous injection of growth complex and estradiol benzoate in the hypophysectomized group closely paralleled weight gains and losses. We have no evidence that it is the sole factor, however, since in no instance did the breasts of these animals approach the degree of proliferation noted in intact animals treated with estrogen alone. It seems, therefore, that the hypothesis formulated by the other workers that the sex hormones act through the pituitary to produce typical changes in the breast is a likely possibility for at least a part of the changes.

We have no explanation for the wide variations in weight in the hypophysectomized animals which were treated at the same time with identical doses of growth complex and estradiol benzoate. Although no microscopic examination was made, the pituitary fossa was examined carefully at autopsy in each instance for evidence of fragments of the anterior pituitary gland. As has been stated above, many of the animals gained or maintained their initial weight after the administration of growth complex. After about 3 weeks, however, they commenced to lose weight rather rapidly. It is reasonable to assume, therefore, that the hypophysis was effectively removed. Several possibilities suggest themselves: (a) The large doses of estrogen may be toxic to the animal; (b) estrogens may inhibit the action of the growth hormone; (c) the animal may become refractory to growth complex. In another experiment we have evidence that the second view is the more tenable. This is also fortified by the work of Zondek, who was able to stunt the growth of immature mice by injecting rather large doses of estrogenic hormone, which probably reduced the anterior pituitary secretion. Experiments to clarify these points are being carried out at present by using much smaller doses of estrogen in combination with the same dose of growth complex.

Conclusion. Nutrition of the animal is an important factor in the effects noted on the breasts of hypophysectomized rats after injection of estrogenic hormone. It is probable, however, that this hormone exerts a part of its effect on the breast by way of the hypophysis.

11008

Natural Vitamin K and Synthetic Vitamin K_1.

S. ANSBACHER, ERHARD FERNHOLZ AND H. B. MACPHILLAMY.
From The Squibb Institute for Medical Research, New Brunswick, N. J.

Several investigators[1-4] described in preliminary form the synthesis of 2-methyl-3-phytyl-1,4-naphthoquinone and its identification with vitamin K_1. In regard to their respective biological ac-

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[1] Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 2557.

[2] Binkley, S. B., Cheney, L. C., Holcomb, W. F., McKee, R. W., Thayer, S. A., MacCorquodale, D. W., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 2558.

[3] Fieser, L. F., *J. Am. Chem. Soc.*, 1939, **61**, 2559.

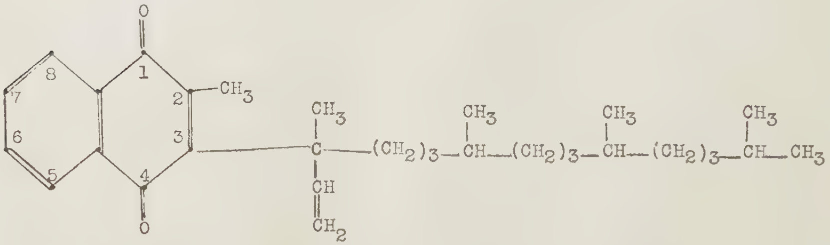
[4] Fieser, L. F., *J. Am. Chem. Soc.*, 1939, **61**, 2561.

tivities, general statements have been made to the effect that both compounds have equal potency.[3-6]

We have prepared 2-methyl-3-phytyl-1,4-naphthoquinone by a modified Fieser[3] procedure with final purification by chromatographic adsorption on calcium sulfate. Using the method of Claisen and Eisleb,[7] we synthesized an isomeric compound, 2-methyl-3-"isophytyl"-1,4-naphthoquinone (Formula I). The biological assays of these compounds are shown in Table I together with those of the Synthetic Vitamin K_1 (Merck),* of the 2-methyl-1,4-naphthoquinone and the diacetate of the corresponding hydroquinone, and of the diacetate of the 2-methyl-3-phytyl-1,4-naphthohydroquinone. The data presented permit the conclusion that the 2-methyl-3-phytyl-1,4-naphthoquinone has a potency of 1 unit[8] in about 7½ γ, its diacetate derivative 1 unit in approximately 15 γ. Furthermore, the Synthetic Vitamin K_1 (Merck), the isomeric compound and 2-methyl-3-dihydrophytyl-1,4-naphthoquinone showed 1 unit in about 15 γ, whereas the methylnaphthoquinone and the diacetate of its corresponding hydroquinone had 1 unit in about ½ γ and 1γ, respectively, as already reported.[9]

The phytyl derivatives appear to have considerably more potency, when the test period is prolonged to 18 hours, as shown in Table II; The speed of action of the unit of 2-methyl-1,4-naphthoquinone is practically identical with that of the unit of the natural vitamin K. However, the phytyl derivatives are not as rapidly utilized by the animal body, as a comparison of Table I with Table II and Table III shows.

FORMULA I.



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[5] MacCorquodale, D. W., McKee, R. W., Binkley, S. B., Cheney, L. C., Holcomb, W. F., Thayer, S. A., and Doisy, E. A., *J. Biol. Chem.* 1939, **130**, 433.

[6] Almquist, H. J., and Klose, A. A., *J. Biol. Chem.*, 1939, **130**, 791.

[7] Claisen, L., and Eisleb, O., *Ann.*, 1913, **401**, 21.

* We wish to take this opportunity of thanking Dr. W. L. Sampson of the Merck Institute for Therapeutic Research for his kindness in sending a sample of Synthetic Vitamin K_1 (Merck).

[8] Ansbacher, S., *J. Nutrition*, 1939, **17**, 303.

[9] Fernholz, E., and Ansbacher, S., *Science*, 1939, **90**, 215.

TABLE I.
Biological Assays.

Dose, γ	No. of chicks	Blood clotting time (minutes)										
		Before treatment (ave)	6 hours after treatment (individual chicks)									
2-Methyl-3-phytyl-1,4-naphthoquinone												
10	5	>90	<1	<1	<2	<2	<2					
7½	10	>90	<2	<2	<3	<4	<4	<5	<5	<6	<8	<10
5	10	>90	<3	<3	<4	<4	<5	<15	<15	>30	>30	>30
2-Methyl-3-"isophytyl"-1,4-naphthoquinone												
15	10	>90	<2	<2	<3	<3	<3	<3	<5	<8	<10	<12
10	9	>90	<2	<2	<4	<8	<10	>30	>30	>30	>30	
Synthetic Vitamin K_1 (Merck)												
15	10	>90	<2	<3	<4	<4	<4	<5	<6	<10	<10	<12
10	10	>90	<3	<4	<5	<5	<7	<7	<10	<21	>30	>30
5	8	>90	<6	<6	<10	<13	>30	>30	>30	>30		
2-Methyl-3-phytyl-1,4-naphthohydroquinone-diacetate												
15	8	>90	<3	<3	<4	<5	<5	<8	<11	<14		
10	10	>90	<3	<3	<4	<6	<9	<13	<15	>30	>30	>30
2-Methyl-1,4 naphthoquinone												
½	10	>90	<2	<2	<2	<2	<3	<3	<4	<5	<5	<6
¼	10	>90	<2	<2	<3	<3	<4	<6	<7	<22	>30	>30
2-Methyl-1,4-naphthohydroquinone-diacetate												
1	10	>90	<2	<2	<3	<3	<4	<5	<5	<5	<8	<13
¾	10	>90	<2	<3	<3	<4	<6	<6	<7	<24	>30	>30

These biological data are particularly interesting, when one considers that we have obtained repeatedly vitamin K concentrates from alfalfa with potencies of 1 unit in about 2 γ,[10] and that these preparations gave a Dam-Karrer color reaction[11, 12] not nearly as strong as the one of 2-methyl-3-phytyl-1,4-naphthoquinone. It seems evident that a vitamin K different from vitamin K_1 exists in alfalfa.

In our experience, concentrates prepared from alfalfa and with a

TABLE II.
Curative Effect

	2-Methyl-3-phytyl-1,4-naphthoquinone 2γ				2-Methyl-3-"Isophytyl"-1,4-naphthoquinone 2γ				Synthetic Vitamin K_1 (Merck) 2γ			
	Blood clotting time (min) of individual chicks after											
Hours	0	6	18	48	0	6	18	48	0	6	18	48
	>90	<5	<1	<2	>90	<6	<2	<10	>90	<4	<1	<3
	>90	<5	<1	<12	>90	>30	<2	<4	>90	<8	<1	>30
	>90	>30	<2	<3	>90	>30	<2	<4	>90	<12	<1	<5
	>90	>30	<2	<6	>90	>30	<2	<7	>90	>30	<2	<3
	>90	>30	<2	>30	>90	>30	<2	>30	>90	>30	<2	<21

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[10] Fernholz, E., Ansbacher, S., and Moore, M. L., *J. Am. Chem. Soc.*, 1939, **61**, 1613.

[11] Dam, H., Geiger, A., Glavind, J., Karrer, P., Karrer, W., Rothschild, E., and Salomon, H., *Helv. Chim. Acta*, 1939, **22**, 310

[12] Karrer, P., *Helv. Chim. Acta*, 1939, **22**, 1146.

TABLE III.
Minimum Effective Doses in 6- and 18-hour Tests.

Substance	6 hr	18 hr	Ratio
	γ	γ	
Vitamin K_1	15	1	15:1
Methylnaphthoquinone	½	¼	2:1
Alfalfa Concentrate	2	1	2:1

potency of 1 unit in about 30 γ are quite complex in composition and contain several chemical individuals with varying vitamin K potencies, as investigations by chromatographic analyses on calcium sulfate show. Some of these fractions are deep red, others are yellow, but our most potent concentrates were always nearly colorless. It should be noted that our process of isolation is different from that employed by other investigators and this may be one of the reasons why we were able to obtain concentrates which had such a high potency but did not give the typical color reaction for vitamin K_1.

11009

Nitrogen Retention on Casein Digests.

WARREN M. COX, JR., AND ARTHUR J. MUELLER.
From the Mead Johnson Laboratories, Evansville, Indiana.

Early work with digests of casein showed that nitrogen retention and growth in young dogs and rats might be promoted[1, 2] by digests prepared by the combined action of digestive juices. In these studies and in those of Hopkins[3] it was recognized that acid digests were incomplete mixtures and had to be supplemented by the addition of tryptophane. Not all workers have been able to confirm these original findings.[4] This may have been due to the quality of the hydrolysates employed. Enzymic hydrolysis, according to Abderhalden's procedure, involves the use of gastric, pancreatic and intestinal extracts, and digestion for a considerable period of time. Boiling hydrochloric or sulfuric acid of the concentration ordinarily used for acid hydrolysis may result in the formation of by-products (amines?) which, in the absence of adequate purification procedures, may mask the actual nutritive value of the digest. A purified casein digest prepared with

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[1] Abderhalden, E., and Oppler, B., *Z. physiol. Chem.*, 1907, **51**, 226; *J. C. S.*, 1907, ii A, 369.

[2] Abderhalden, E., *Z. physiol. Chem.*, 1915, **96**, 1.

[3] Hopkins, F. G., *J. Chem. Soc.*, 1916, **109**, 629.

[4] McClendon, J. F., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 915.

pancreatic enzymes has been made in this laboratory and has been shown to be satisfactory for the growth of rats, and for the regeneration of serum protein in dogs, when assayed in comparison with the unhydrolyzed casein.[5] Acid digests have also been made and carefully purified. Both preparations, after drying, were light, impalpable powders, almost white. On dissolving they gave straw-colored solutions. The present report deals with the nitrogen balance obtained with both types of digest.

Experimental. Adult male rats were placed in metabolism cages adapted to separate urine from feces and to minimize spilling of the food. The rats were first placed on stock diet until they became accustomed to the cage and were then transferred to the nitrogen-low diet. The composition of the basal diet was:

Maltose and Dextrins*	57.0
Olive Oil	22.7
Starch (arrowroot)	12.4
Salts†	7.9

The various ingredients were mixed with water, heated to boiling, homogenized and dried in a pilot scale spray-drier. Analysis of the powder showed the following percentage composition: protein, 0.25; fat, 23.04; ash, 4.60; moisture, 0.81; carbohydrates (by difference), 71.30.

Necessary vitamins were supplied by one yeast tablet (.39 g, containing approximately 20 I.U. of B_1 and 20 Sherman units B_2) on which was placed one drop of Oleum Percomorphum daily. The total nitrogen intake from the basal diet and vitamin supplements was between 30 and 38 mg per day.

Feces and urine were collected daily and separately; food and water intake was carefully measured. Feces were prepared for analysis by grinding, and treatment with sulfuric acid according to the method of MacKay and Butler.[6] All nitrogen determinations were made in duplicate by micro-Kjeldahl.

Comparison of Casein and Enzymic Casein Digest. In Fig. 1, 68 daily nitrogen balances for 7 periods of observation are graphically presented. The fecal and urinary nitrogen excretions are indicated by cross-hatched and white areas on the lower half of the chart. The

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[5] Mueller, A. J., Kemmerer, K. S., Cox, W. M., Jr., and Barnes, S., in press.

* Dextri-Maltose.

† Calcium gluconate, 4.0; Monobasic potassium phosphate, 1.9; Calcium hydroxide, 1.0; Potassium chloride, 0.5; Dibasic potassium phosphate, 0.5; Magnesium oxide, 0.05.

[6] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins, Baltimore, 1932.

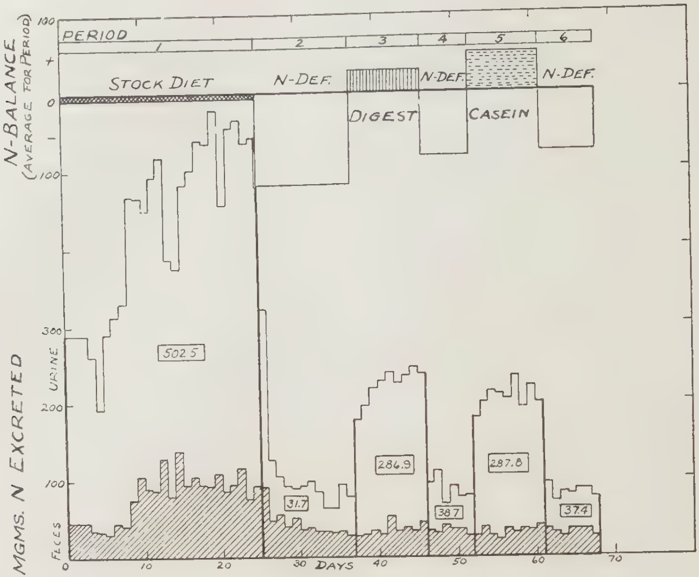

FIG. 1.

Daily nitrogen excretion and nitrogen balance of adult male rat receiving stock diet (Period 1) and a nitrogen-free basal diet (Periods 2, 4 and 6) supplemented with an enzymic digest of casein (Period 3) and casein (Period 5).

average nitrogen balances for the period are given graphically at the top and the nitrogen intake numerically in blocked squares. During Period 1, stock diet *ad libitum,* food intake was initially quite low, and even though large positive nitrogen balances were obtained after the rat had become accustomed to the surroundings, the average balance for the 25 balance periods was –8.7 mg/day. Large negative balances followed the substitution of the basal, nitrogen-low diet (Period 2) with prompt drop in both urinary and fecal excretion of nitrogen.‡

The enzymic digest of casein (92-Z) prepared as described elsewhere,[5] was mixed with a small amount of the basal diet and avidly consumed by the rat during Period 3. The nitrogen balance was positive (27 mg/day), and the urinary and fecal excretion remarkably constant throughout the period. In order to facilitate the comparison only 2 g of the digest (251 mg N) was fed, although the nitrogen intake on stock diet averaged about 500 mg N per day. The total nitrogen intake was, therefore, approximately half of the usual intake.

Acid-washed, vitamin-free casein was fed at the same nitrogen intake level, and, as will be noted (Period 5), with slightly greater nitrogen retention (49 mg/day). The urinary excretion of nitrogen was approximately the same as when the digest was fed. The conclusion seems warranted that both casein and an enzymic digest of casein prepared as described, result in positive nitrogen retention.

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‡ The nitrogen intake during the first period on the basal diet is lower than on later periods because for the first few days, until nitrogen deficiency was prominent, the yeast tablet was not all eaten.

Retention on Acid and Enzymic Casein Digests. Acid hydrolysis of protein results in the complete destruction of tryptophane, and the resulting mixture of amino acids is nutritionally inadequate. It was desirable, however, to determine if any of the nitrogen of such a preparation might be retained or whether feeding such a preparation would be equivalent to subsistence on a low-nitrogen diet. Evidence of decreased nitrogen loss and even of slight positive retention on nutritionally inadequate mixtures of amino acids has recently been presented by Nielsen and Corley.[7]

Fig. 2 shows the nitrogen retention on the enzymic digest (Period 2) and on the acid digest (89) supplemented with 0.2% tryptophane (Periods 4 and 6). Both supplements were greedily consumed by the animal. Following Period 4, and without an intervening period on the basal diet, the tryptophane was omitted. On the first day all the supplement was again consumed (with a resultant increase in

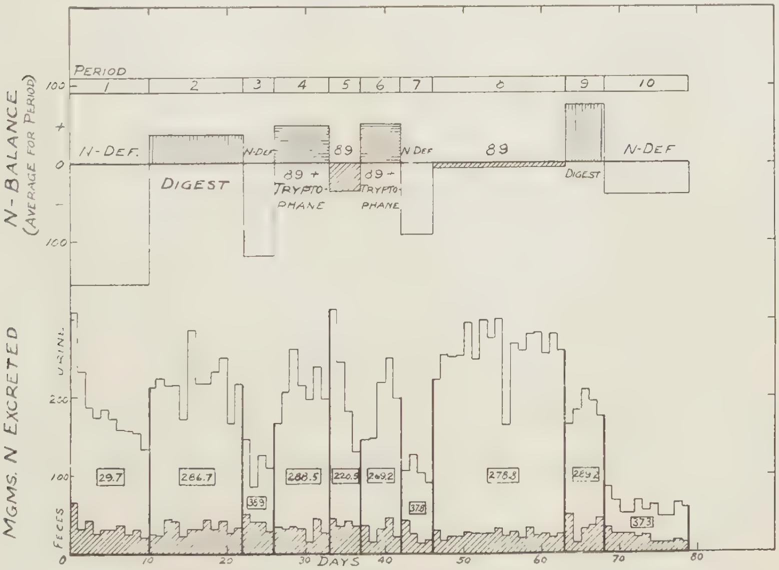

FIG. 2.

Daily nitrogen excretion and nitrogen balance of adult male rat receiving a nitrogen-free basal diet (Periods 1, 3, 7 and 10) supplemented with an enzymic digest of casein orally (Period 2), and by stomach tube (Period 9); with an acid digest of casein orally (Period 5) and by stomach tube (Period 8); and the latter plus tryptophane (Period 4 and 6).

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[7] Nielsen, E. K., and Corley, R. C., *J. Physiol.*, 1939, **126**, 223.

urinary nitrogen), but on the subsequent 3 days progressively less was eaten until on the fourth day only 91 of the 250 mg nitrogen offered was ingested. This decrease in intake is indicated by the rapid drop in urinary excretion during Period 5.

Tryptophane was then added to the supplement and after 2 days of cautious approach (indicated here by low urinary excretion), it was again eaten greedily, with resultant positive balances. This ability of the animal to distinguish between nitrogen sources that are adequate and those that are inadequate has been frequently observed.[8] The daily urinary excretions, however, give an excellent picture of the rapidity with which an animal can adjust for changes in adequacy.

To get a true comparison, it was necessary to administer the acid digest by stomach tube (Period 8). Half of the total amount (2 g = 250 mg N) was given in the morning and half in the afternoon. Daily nitrogen balances were run for 17 days. As will be noted (Period 8), urinary excretion was quite constant (on the tenth day only half of the required amount was given) and there was a small average negative nitrogen balance for the period. The magnitude of the loss was considerably less than on the nitrogen-low basal diet alone, and would indicate that a considerable portion of the acid hydrolysate was utilized or stored by the body. During this 17-day period, weight declined from 356 to 339 g for an average loss of one gram per day, indicating that in spite of the approximate balance in nitrogen metabolism, the mixture was far from nutritionally adequate. While this is to be interpreted as a severe weight loss, it was not so severe as that recorded on the basal diet. There were 4 such periods (1, 3, 7, 10) and the daily weight loss for each respectively was 7.1, 3.7, 2.0, and 2.9 g. The animal thus fared better than if no form of nitrogen had been given. It might be assumed that the small amount of tryptophane from the vitamin supplement and possibly in the basal diet was the factor promoting a partial utilization of the incomplete acid hydrolysate.

This period was followed immediately by a period of 5 days during which an identical amount of nitrogen, in the form of the enzymic hydrolysate, was given by stomach tube (Period 9). Several things of interest are to be noted: weight was stationary at 337 g, the animal went into large positive nitrogen balance, and the urinary excretion of nitrogen declined sharply. The picture is entirely different from that obtained with the acid hydrolysate, and suggests that all essential amino acids were present in the digest prepared by enzymic action.

Summary. 1. Continuous daily nitrogen balances covering 140 days have been made on rats restricted to a nitrogen-low basal diet, supplemented for various periods with casein, and an acid or an enzymic digest of casein. 2. The enzymic digest fed in comparison with casein, resulted in nitrogen retention of approximately the same magnitude as the unhydrolyzed protein. 3. The acid digest, supplemented with 0.2% tryptophane likewise gave positive nitrogen balances, but without the addition of the essential tryptophane, was refused by the animals. 4. When both the unsupplemented acid digest, and the enzymic digest were administered by stomach tube, the latter was well retained, but the former gave a slight negative balance with some indication that it was partially effective in supplying animal requirement for nitrogen.

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[8] Rose, W. C., *Physiol. Rev.*, 1938, **18**, 109.

11010

Quantitative Biological Assay of Vitamin K and Its Application to Several Quinone Compounds.*

Flemintine Peirce Dann. (Introduced by Carl Nielsen.)
From the Abbott Laboratories, North Chicago, Illinois.

The Biological Assay Technic. When this work was started in 1937, the 2 methods used for the biological assay of the antihemorrhagic factor were the curative technic of Schønheyder[1] and the preventive method of Almquist and Stokstad.[2] The Schønheyder method which depends on a curative effect is theoretically more accurate than a preventive one. In the application of the method, the long and involved technic of taking blood samples and determining the blood clotting time is a definite disadvantage. Later, Dam and Glavind[3] revised the Schønheyder method without simplifying these steps.

In the meantime, some work[4] had been done in our laboratory on the Almquist and Stokstad preventive method. Finally, a method for the biological assay of the antihemorrhagic factor was developed, using the diet and blood-clotting technic of Almquist and Stokstad, the 3-day curative period of Dam, *et al.*, the oral administration of the vitamin K test dose to the chicks individually, and a vitamin K standard as a positive control for each group of chicks. This curative method which has been used with the vitamin K standard for a year and a half is quantitative within the limits of error for a biological assay, provided the procedure is carried out with certain precautions. Chicks are obtained from a source where the diet of the hens is standardized and is the same the year round. The fish meal and yeast in the deficient diet is continuously extracted with ether until the ether wash no longer contains any fat. The wire bottoms and cages are kept very clean throughout the assay period to prevent coprophagy. The incision of the main wing vein is made with a flexible razor blade, and not less than 0.5 cc of blood is withdrawn. The blood clotting time of 10 or more chicks is determined before the assay is started; if 70% or more of these negative controls do not show a clotting time of over 30 minutes, the chicks are continued on the deficient diet and the clotting time is checked every few days until the chicks are depleted of vitamin K. For a finished report, not less than 20 chicks are used to a level and the standard is run concurrently with the unknown. The potency is calculated in vitamin K units per gram by the formula, dose of standard/dose of unknown × units per gram in the standard. When the potency of 5000 vitamin K units per gram was assigned to our standard, a dose of 0.2 mg fed per chick per day gave a blood clotting time of approximately 2 minutes. Since then our method of assay and technic of taking blood clotting time have been modified. Essentially the same procedure has been followed with other standards for biological assay work, namely, arbitrarily assigning a definite potency to a standard and retaining the same factor although later the original assay method was modified. The standard selected in this case was a vitamin K oil concentrate prepared from alfalfa with the hexane and most of the sterols removed. For oral administration, it is diluted with sesame oil and 0.1 cc measured by a syringe into gelatin capsules, the unknown being fed in the same way. This standard has proved to be a workable and stable material. The data in Table I show no difference in potency of a sample of our standard kept in a screw-top bottle without nitrogen at 40°C for 14 months and one stored at 5°C for the same period.

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* The author is indebted to Dr. H. Dam for his standard spinach tablets, to Dr. R. J. Anderson for the phthiocol compound, to Dr. Byron Riegel for a sample of the natural vitamin K_1, to Dr. E. A. Doisy for a specimen of his natural vitamin K_1, and to Dr. L. F. Fieser for a sample of the synthetic vitamin K_1.

[1] Schønheyder, F., *Biochem. J.*, 1936, **30**, 890.

[2] Almquist, H. J., and Stokstad, E. L. R., *J. Nutri.*, 1937, **14**, 235.

[3] Dam, H., and Glavind, J., *Biochem. J.*, 1938, **32**, 1018.

[4] Dann, F. P., *Am. J. Physiol.*, 1938, **123**, 48.

Discussion of Assay Methods. During the last year, many assay technics have been suggested and later modified. The described 3-day curative method is the only one which has been consistently adhered to so that enough data have accumulated to check the ac-

TABLE I.
Correlation of Standard A and the Dam Standard with Data on Stability of Standard A.

Material	Daily dose, mg	No. of chicks	Avg clotting time, min
Vitamin K Std. A—stored at 5°C	0.2	22	7.96 ± 1.05*
" " " " " " 40°C	0.2	21	7.86 ± 1.08
Std. Spinach Tablets—Dam	50.0	10	7.27 ± 1.23
" " " "	75.0	22	4.65 ± 0.39†
Negative controls		20	More than 25.66

* On this dose level, the standard deviation is great. The most sensitive level for accurate interpretation of results is the 75 mg dose on the Dam standard with a percentage error of approximately 10%,† or a dose level of 0.30 mg on Standard A and 0.45 mg on Standard B which is ⅔ as active as A.

curacy of the technic. In the meantime, other methods were tried without any increase in accuracy. Almquist, Mecchi and Klose[5] modified their original preventive method and suggested from 1 to 2 weeks' curative period. This method in which the chicks are placed on test after only 1 week on the vitamin K-free diet and therefore do not reach a state of avitaminosis, is not precisely a curative technic. It is based on the assumption, as is the preventive method, that the vitamin K reserve is the same in each group of chicks, which is not the case. Recently, Almquist and Klose[6] modified the technic of taking the simple blood clotting time by determining the whole blood prothrombin time. In checking this method in our laboratory negative controls which had a blood clotting time of over 30 minutes showed a prothrombin time of from 47 to 93 seconds, while the normal chick's prothrombin time was 25 to 37. Such a close relationship between the deficient and the normal chicks produces slight differences in dose responses, decreasing the assay's sensitivity.

Thayer, *et al.*,[7] have reported on the 3-day curative method using a dose response curve for interpretation of results and a unit defined as that quantity of vitamin which produces a clotting time of 10 minutes or less in 50% of a group of 10 or more chicks. Such a dose level is very close to a borderline level where the individual variation is great. On the other hand, the calculated standard deviation will not give an accurate picture of this variation where the clotting time is not observed after 30 minutes. Later, Thayer, *et al.*,[8] modified their method to an 18-hour test, while Ansbacher[9] reduced the test period to 6 hours. In our hands, these short procedures work very nicely for preliminary research work where one is only interested in approximate potencies but are not accurate enough for quantitative work.

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[5] Almquist, H. J., Mecchi, E., and Klose, A. A., *Biochem. J.*, 1938, **32**, 1897.

[6] Almquist, H. J., and Klose, A. A., *Biochem. J.*, 1939, **33**, 1055.

[7] Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 478.

[8] Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 194.

[9] Ansbacher, S., *J. Nutri.*, 1939, **17**, 304.

The Vitamin K Activity of Several Quinone Compounds. The earliest chemical studies on vitamins K_1 and K_2 indicated that they contained the quinone structure.[10] The vitamin K activity of the pure substances and the related quinone compounds was reported by different workers.[11] These reports were conflicting, partly due to the assays being preliminary and partly due to the different methods and units used. Data collected in this laboratory on the vitamin K activity of these compounds in terms of 1 unit may help to clarify the confusion in this field.

The two compounds which first created the most interest in the recent literature were the 2-methyl-3-hydroxyl-1,4-naphthoquinone known as phthiocol and the 2-methyl-1,4-naphthoquinone. Preliminary assays were first run on these compounds and later were reassayed by the 3-day curative method with a greater number of chicks to a level. The activity of the phthiocol in an oil medium is on the borderline of 15 units per mg; the sodium salt in a saline solution is definitely not as active as the original compound in oil, being less than 10 units per mg. The 2-methyl-1,4-naphthoquinone contains 2500 units per mg when administered orally in an oil solution. (Table III.) The first sample of this compound with a m.p. of 100-101°, assayed 2250 units; later a slightly purer sample, m.p. 104-106°, assayed 2500. The activity is significantly less with other solvents and different methods of administration (Table II). The reason for this is not clearly understood at present.

Dr. Marjorie B. Moore of our research staff first suggested the

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[10] Dam, H., Geiger, A., Glavind, J., Karrer, D., Karrer, W., Rothschild, E., and Soloman, H., *Helv. chim. Acta,* 1939, **22**, 310; McKee, R. W., Binkley, S. B., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1295; Binkley, S. B., MacCorquodale, D. W., Cheney, L. C., Thayer, S. A., McKee, R. W., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1612.

[11] Thayer, S. A., Cheney, L. C., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1932; Karrer, P., and Geiger, A., *Helv. chim. Acta*, 1939, **22**, 945; Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 1611; Fieser, L. F., Bowen, D. M., Campbell, W. P., Frey, E. M., and Tates, M. D., *J. Am. Chem. Soc.*, 1939, **61**, 1926; Ansbacher, S., and Fernholz, E. J., *J. Am. Chem. Soc.*, 1939, **61**, 1924; Thayer, S. A., Binkley, S. B., MacCorquodale, D. W., Doisy, E. A., Emmett, A. D., Brown, R. A., and Bird, O. D., *J. Am. Chem. Soc.*, 1939, **61**, 2563; Tishley, M., and Sampson, W. L., *J. Am. Chem. Soc.*, 1939, **61**, 2563; MacCorquodale, D. W., McKee, R. W., Binkley, S. B., Cheney, L. C., Holcomb, W. F., Thayer, S. A., and Doisy, E. A., *J. Biol. Chem.*, 1939, **130**, 433; Almquist, H. J., and Klose, A. A., *J. Biol. Chem.*, 1939, **130**, 787.

TABLE II.
Vitamin K Activity of 2-methyl-1,4-naphthoquinone.

Daily dose, micro-grams	Solvent	Method of admin.	Avg clotting time	Dose of Std. B, micro-grams	Avg clotting time	Potency in units per mg
0.6	Oil	Oral	4.44	450	4.87	2500
0.6	”	Intramus.	4.78	450	4.87	2500
2.0	Aqueous	Oral	5.38	450	5.40	750
1.5	”	Intramus.	4.93	450	5.40	1000
1.0	Propylene Glycol	”	5.51	450	5.62	1500

TABLE III.
Vitamin K Activity of Vitamin K_1 and Other Quinone Compounds.

Material*	Daily dose, micro-grams	Avg clotting time	Dose of Std. B, micro-grams	Avg clotting time	Potency in units per mg
Synthetic K_1	2.00	4.86	450	4.69	750
Natural K_1	1.50	4.18	450	4.26	1000
2-methyl-1,4-naphthoquinone	0.60	4.44	450	4.87	2500
2-methyl-1,4-naphthoquinhydrone	0.60	5.12	450	4.87	2500
2-methyl-1,4-naphthohydroquinone	0.75	7.16	450	4.35	<2000

* All these materials were dissolved in sesame oil and 0.1 cc fed orally per chick per day.

possibility of 2-methyl-1,4-naphthoquinhydrone having vitamin K activity. Later she found that this compound had been synthesized once before by the Spanish workers, Madináveitia and de Buruaga.[12] A sample of this compound with a m.p. of 106-109° (this was not a sharp m.p.) assayed 2500 units per mg (Table III). The 2-methyl-1,4-naphthohydroquinone, m.p. 158-159° with decomposition, contains slightly less than 2000 units per mg. In comparison with the vitamin K activity of these synthetic compounds, the natural vitamin K_1, $C_{31}H_{46}O_2$ (found: C, 82.42 H, 10.41) showed a potency of 1000 units per mg (Table III). The question arises whether this sample of natural vitamin K_1, which is very sensitive to light, was slightly inactivated. Later, a second specimen of this material carefully protected from light throughout the procedure, was assayed and checked the first results nicely. The sample of synthetic vitamin K_1 (found: C, 82.40 H, 10.44) was slightly less active than the natural K_1, being 750 units per mg. The molecular weight of vitamin K_1 is 2.6 times that of 2-methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinhydrone, hence the compounds are approximately equally active on a molar basis.

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Conclusions. (1) A 3-day curative technic for the biological assay of the antihemorrhagic factor has been developed. If a vitamin K standard is run with each group of chicks and certain precautions in the procedure are observed, the assay is quantitative within the limits of error for a biological assay. (2) The vitamin K activity of a compound varies significantly with the solvent and the method of administration. (3) The vitamin K activity of 2-methyl-1,4-naphthoquinone and of 2-methyl-1,4-naphthoquinhydrone is approximately the same as the natural vitamin K_1 on a molar basis.

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Action of Sulfanilamide on Hemolytic Enterococcus (*Streptococcus fecalis hemolyticus*).

ERWIN NETER.

From the Laboratories of the Children's Hospital, and the Department of Pathology and Bacteriology, University of Buffalo School of Medicine, Buffalo, N. Y.

It is generally agreed that sulfanilamide, sulfapyridine, and related compounds are effective in the treatment of infections due to a limited number of microörganisms only. *In vitro,* these chemotherapeutic substances may be bacteriostatic or bactericidal toward susceptible strains. There is, however, no absolute parallelism between the effectiveness of sulfanilamide *in vivo* and *in vitro;* nor between susceptibility or resistance of certain species or groups of microörganisms and their respective position in the system of bacteria. The susceptibility toward the action of sulfanilamide may be quite different even among relatively closely related groups. Thus, sulfanilamide may inhibit the growth of Lancefield Group A hemolytic streptococci in broth, while strains of hemolytic streptococci Group D (hemolytic enterococci) are quite resistant toward this drug (Bliss and Long[1]). Recently, it was reported[2, 3] that sulfanilamide in a concentration of 800 mg per 100 ml may continuously inhibit the growth of fibrinolytic hemolytic streptococci even when relatively large numbers were used for inoculation; in contrast, strains of hemolytic enterococci, characterized by their growth on 40% bile agar, by their capacity to reduce methylene blue and litmus in skimmed milk, by their production of a pH below 4.8 in 1% glucose broth, and by their ability to ferment mannitol, were only slightly or not at all inhibited in 0.8% sulfanilamide broth. The culture medium used in these experiments was phenol-red broth (Difco), to which 1% soluble starch, 0.2% agar, and 1% maltose or dextrose, respectively, were added. Subsequently, it was found that essentially the same results were obtained when starch and agar were omitted from this medium. These observations are in agreement with the findings of Long and Bliss,[4] who showed that streptococci of Group D are resistant toward the bacteriostatic action of sulfanilamide in concentrations up to 0.8%, while in higher concentrations this drug slightly retarded the growth of a few strains. It was noted that the strains of hemolytic enterococci at our disposal grew more rapidly in the above described phenol-red broth than did Group A hemolytic streptococci; visible growth occurred within a few hours following inoculation of the medium. Observations[5–10] of the so-called "lag-period" in the action of sulfanilamide and sulfapyridine, both *in vivo* and *vitro,* suggested that the rapid growth of hemolytic enterococci may in part be responsible for the negligible bacteriostatic action of sulfanilamide toward these strains under the above-reported conditions. Sherman[11] showed that both hemolytic enterococci *(Streptococcus zymogenes* and *Streptococcus durans)* and non-hemolytic enterococci *(Streptococcus fecalis* and *Streptococcus liquefaciens)* may grow in broth containing 6.5% sodium chloride in contradistinction to hemolytic streptococci Groups A, B, and C. Employing this test for differential diagnostic purposes, it was noted that the growth of hemolytic enterococci in this medium was much slower than that in broth containing only 0.5% sodium chloride. This retarding influence of sodium chloride on the growth of hemolytic enterococci was utilized in the following experiments dealing with the action of sulfanilamide on hemolytic enterococci in broth containing 6.5% sodium chloride.

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[1] Bliss, S. A., and Long, P. H., *New England J. Med.*, 1937, **217**, 18.

[2] Neter, E., *J. Bact.*, 1938, **36**, 669.

[3] Neter, E., *J. Lab. and Clin. Med.*, 1939, **24**, 650.

The medium was prepared as follows: Phenol-red broth (Difco) containing tryptose, 0.5% sodium chloride, 0.1% dipotassium phosphate, and phenol red as indicator was dissolved in the given amount in neutral distilled water; then the respective amounts of maltose and sodium chloride were added. Sulfanilamide (purified) in the amount of 0.8% was added to one part of this medium, while the other part was used as control. The broths were autoclaved at 15 pounds pressure for 12 minutes; the medium was used only if no noticeable change in the pH occurred following this procedure.

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[4] Long, P. H., and Bliss, E. A., *The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine, and Allied Compounds,* The Macmillan Company, 1939, 102.

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[7] Osgood, E. E., *J. Am. Med. Assn.,* 1938, **110**, 349.

[8] Lockwood, J. S., *J. Immunol.,* 1938, **35**, 155.

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[10] McIntosh, J., and Whitby, L., *Lancet,* 1939, **1**, 431.

[11] Sherman, J. M., *Bact. Rev.,* 1937, **1**, 16.

Four strains of hemolytic enterococci were used. Two of these were obtained through the courtesy of Dr. J. M. Sherman, Ithaca, New York. For inoculation, 0.1 cc of a 2- to 5-hour brain-heart-infusion broth-culture or dilution thereof, was added to about 5 cc of each sulfanilamide and control broth. The tubes were incubated at 37°C; growth and acid-production were noted at various intervals.

Table I presents the results of an experiment on the bacteriostatic action of sulfanilamide (0.8%) on a strain of hemolytic streptococcus Group A and a strain of hemolytic enterococcus (Group D) in 1% maltose phenol-red broth containing 0.5% and 6.5% sodium chloride, respectively. It may be seen, that: (1) sulfanilamide in broth containing 0.5% sodium chloride retarded the growth of the hemolytic enterococcus only to a slight degree, while it completely and continuously inhibited the growth of Group A hemolytic streptococcus; (2) the growth of the hemolytic enterococcus was markedly delayed in broth containing 6.5% sodium chloride, while Group A hemolytic streptococcus failed to grow; (3) sulfanilamide (0.8%) in broth containing 6.5% sodium chloride prevented visible growth of the hemolytic enterococcus for 2 days and later inhibited the resulting growth. This bacteriostatic action of sulfanilamide on hemolytic enterococcus was repeatedly demonstrated with 4 strains at our disposal—provided that a suitable number of bacteria were used for inoculation. In a number of experiments, sulfanilamide in concentration of 1% completely prevented visible growth of the hemolytic enterococcus, even when incubation was carried out for one week. It must be emphasized, however, that the results of these experiments on the bacteriostatic action of sulfanilamide on hemolytic enterococci were not as striking and uniform as those obtained with fibrinolytic hemolytic streptococci.

Preliminary experiments indicate that the degree of effectiveness of sulfanilamide on the growth of hemolytic enterococci is linked to the carbohydrate-content of the medium. Thus, in the presence of 1% dextrose, the bacteriostatic action of sulfanilamide toward these strains was negligible. On the other hand, when the maltose-content of the medium was reduced from 1% to ¼%, sulfanilamide in a concentration of 1% definitely inhibited the growth of small numbers of

TABLE I.

Bacteriostatic Action of Sulfanilamide on *Streptococcus hemolyticus* and *Enterococcus hemolyticus*

Hrs Incubation 37°C	Growth and acid-production by I *Streptococcus hemolyticus* in phenol-red maltose broth containing				II *Enterococcus hemolyticus*			
	a	b	c	d	a	b	c	d
	.5% NaCl	.5% NaCl + .8% Sulfanilamide	6.5% NaCl	6.5% NaCl + .8% Sulfanilamide	.5% NaCl	.5% NaCl + .8% Sulfanilamide	6.5% NaCl	6.5% NaCl + .8% Sulfanilamide
2	+	—	—	—	+	—	—	—
8	+++	—	—	—	++++	+++	+	—
18	++++	—	—	—	++++	+++	++	—
48	++++	—	—	—	++++	+++	+++	—
72	++++	—	—	—	++++	+++	+++	+

— = no growth and no acid-production.
+ to ++++ = various degrees of growth and acid-production.

hemolytic enterococcus, even when the sodium-chloride content of the medium was only 0.5%.

The demonstration of the bacteriostatic action of sulfanilamide in high concentrations on hemolytic enterococci in phenol-red broth containing from 6.5 to 7% sodium chloride, may possibly be explained on the basis of Mellon's[12] observation of a potentiating effect of sodium chloride and sulfanilamide. Or, the possibility may be considered that the retardation of growth by sodium chloride may overcome the lag-period necessary for the action of this drug; this latter point of view, however, leaves unexplained the fact that growth may occur after several days of incubation. The foregoing experiments make it seem possible that other changes in the environmental conditions may enhance the bacteriostatic action of sulfanilamide toward species and groups of microörganisms that thus far were considered to resist the action of the drug.

In conclusion: (1) Sulfanilamide in concentrations of 0.8% to 1% in 1% maltose phenol-red broth retarded the growth of hemolytic enterococci to a slight degree only, while it continuously inhibited the growth of fibrinolytic hemolytic streptococci. (2) A more marked bacteriostatic action of sulfanilamide (0.8 to 1%) on small numbers of hemolytic enterococci could be demonstrated when the sodium-chloride content of this medium was increased to 6.5% or 7%, or when the maltose-content was decreased from 1% to ¼%.

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[12] Mellon, R. R., Gross, P., and Cooper, F. B., *Sulfanilamide Therapy of Bacterial Infections,* Charles C. Thomas, 1938, 233.